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(54) Title: HUMAN AND MOUSE β-DEFENSINS, ANTIMICROBIAL PEPTIDES

(57) Abstract: The present invention employs an iterative application of BLAST and Hidden Markov Model (HMM) based searches which identified 34 β-defensin genes in the human genome and 48 in the mouse genome. The present invention relates to novel antimicrobial peptides and derivatives thereof as well as the β-defensin genes encoding the peptides. The invention further relates to methods of use of the peptides including a method of inhibiting microbial growth by administering an effective amount of the peptide alone or in combination with other antimicrobial agents antibiotics.

HUMAN AND MOUSE β-DEFENSINS, ANTIMICROBIAL PEPTIDES

BACKGROUND OF THE INVENTION

The government owns rights in the present invention pursuant to grant number HL-61234 from the National Institutes of Health. This application claims benefit of priority to U.S. Serial No. 60/323,991, filed September 21, 2001, the entire contents of which is hereby incorporated by reference without reservation.

1. Field of the Invention

This invention relates generally to antimicrobial agents and to methods of preventing microbial growth. In particular, the present invention involves compositions comprising an antimicrobial peptide and methods for its use.

2. Description of Related Art

The first antibiotics were used clinically in the 1940s and 1950s, and their use has been increasing significantly since this period. Although an invaluable advance, antibiotic and antimicrobial therapy suffers from several problems, particularly when strains of various bacteria appear that are resistant to antibiotics. Interestingly, bacteria resistant to streptomycin were isolated about a year after this antibiotic was introduced.

Antibiotic resistance is a serious and life-threatening event of worldwide importance. For example, strains of *Staphylococcus* are known that are immune to all antibiotics except one (Travis, 1994). Such bacteria often cause fatal hospital infections. Other drug resistant organisms are pneumococci that cause pneumonia and meningitis; *Cryptosporidium* and *E. coli* that cause diarrhea; and enterococci that cause blood-stream, surgical wound and urinary tract infections (Berkelman *et. al.*, 1994). The danger is further compounded by antibiotic and antimicrobial resistance, which may spread vertically and horizontally by plasmids and transposons.

Davies (1986) described seven basic biochemical mechanisms for naturally-occurring antibiotic resistance: (1) alteration (inactivation) of the antibiotic; (2) alteration of the target site; (3) blockage in the transport of the antibiotic; (4) by-pass of the antibiotic sensitive-step (replacement); (5) increase in the level of the inhibited enzyme (titration of drug); (6) sparing the

antibiotic-sensitive step by endogenous or exogenous product; and (7) production of a metabolite that antagonizes action of inhibitor.

Antimicrobial peptides have been isolated from plants, insects, fish, amphibia, birds, and mammals (Gallo, 1998; Ganz & Lehrer, 1998). Vertebrate skin, trachea and tongue epithelia are rich sources of these peptides, which may explain the unexpected resistance of these tissues to infection (Russell *et al.* 1996). Although previously considered an evolutionarily primitive system of immune protection with little relevance beyond minimal antimicrobial activity, it has subsequently been determined that antimicrobial peptides are a primary component of an innate immune response and are expressed by mammalian cells during inflammatory events such as wound repair, contact dermatitis and psoriasis (Nilsson, 1999). The efficacy of antimicrobial peptides is based upon their ability to create pores in the cytoplasmic membrane of microorganisms (Oren *et al.*, 1998). They also have been shown to stimulate syndecan expression, chemotaxis, and chloride secretion (Gallo, 1998).

The present invention seeks to employ antimicrobial compounds to overcome the deficiencies inherent in the prior art by providing new compositions, combined compositions, methods and kits, for treating infections and reducing resistance to antimicrobials and antibiotics.

SUMMARY OF THE INVENTION

Thus, in accordance with the present invention, there is provided an isolated antimicrobial peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOS:1-82. The antimicrobial peptide may be comprised in a pharmaceutically acceptable composition, for example, one suited for topical, parenteral or oral administration. The pharmaceutical composition may, in particular, be formulated for administration by injection or inhalation.

In another embodiment, there is provided an isolated nucleic acid molecule encoding a peptide selected from the group consisting of SEQ ID NOS:1-82, the nucleic acid molecule isolated free from other human or murine coding sequences. The nucleic acid molecule may be incorporated into an expression vector. In yet another embodiment, there is provided a viral vector comprising a nucleic acid molecule encoding a peptide selected from the group consisting of SEQ ID NOS:1-82. The viral vector may be selected from the group consisting of adenovirus, adeno-associated virus, vaccinia virus, retrovirus, herpesvirus, and polyomavirus.

In still yet another embodiment, there is provide an isolated nucleic acid molecule encoding a peptide selected from the group consisting of SEQ ID NOS:1-82, and a promoter heterologous to the coding region for the peptide. The promoter may be CMV IE. The isolated

nucleic acid molecule may further comprise one or more of an origin of replication, a polyadenylation signal, an internal ribosome entry site, a multipurpose cloning site and a selectable marker.

In yet a further embodiment, there is provided an isolated nucleic acid molecule encoding a peptide selected from the group consisting of SEQ ID NOS: 1-82, the nucleic acid molecule being 10,000 base pair in length or shorter. The isolated nucleic acid molecule may 5000 base pairs or shorter, 2500 base pairs or shorter, 1000 base pairs or shorter, or 500 base pairs or shorter.

In still yet a further embodiment, there is provided a method of inhibiting the growth of a microbe comprising introducing into an environment containing the microbe a peptide selected from the group consisting of SEQ ID NOS:1-82. The peptide may be introduced in a composition capable of sustaining the antimicrobial properties of the peptide in the environment, such as a pharmaceutical composition. The method may further comprise introducing an additional antimicrobial agent into the environment. The peptide may be introduced before the additional antimicrobial agent, after the additional microbial agent, or the peptide and the additional antimicrobial agent may be introduced concurrently. The additional antimicrobial agent may be a protein synthesis inhibitor, a cell wall growth inhibitor, a cell membrane synthesis inhibitor, a nucleic acid synthesis inhibitor, and a competitive inhibitor. The environment may be a surgical field or wound site.

In an additional embodiment, there is provided a kit comprising an antimicrobial peptide, wherein the peptide comprises an amino acid sequence selected from the group consisting of SEQ ID NOS:1-82, disposed in a suitable container. The kit may further comprise an additional antimicrobial agent.

Another embodiment comprises a method of inhibiting growth of a microbe in a host, comprising administering to the host a peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOS:1-82. The method may further comprise administering an additional antimicrobial agent, before, after or at the same time as adminstration of the peptide. The additional antimicrobial agent may be a protein synthesis inhibitor, a cell wall growth inhibitor, a cell membrane synthesis inhibitor, a nucleic acid synthesis inhibitor, and a competitive inhibitor.

In still an additional embodiment, there is provided a medical device coated with one or more peptides selected from the group consisting of SEQ ID NOS:1-82. The device may be a catheter, a needle, a sheath, or a stent.

Addition embodiments include an antimicrobial composition comprising one or more peptides selected from the group consisting of SEQ ID NOS:1-82 and one or more non-peptide antimicrobial agents; a method of treating a bacterial infection comprising administering to a subject a peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOS:1-82; a method of activating a memory T cell comprising contacting a memory T cell with a peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOS:1-82; a method of activating an immature dendritic cell comprising contacting an immature dendritic cell with a peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOS:1-82; a method of stimulating adaptive immune response comprising contacting a subject with a peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOS:1-82; a method of inhibiting a multidrug resistant bacterium comprising treating the bacterium with a peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOS: 1-82

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1: Multiple sequence alignment of predicted human ß-defensin proteins. The amino acid sequences were predicted from the genomic sequence of the indicated ß-defensin gene. The Genbank accession numbers for the genomic sequence is available in Appendix 1. The genomic location of each gene is indicated. The location of the DEFB30 and DEFB31 genes is ambiguous as they map to multiple and different locations in the sequence of the human genome. ß-defensin genes encode predicted amino acid sequences that contain a six-cysteine motif with the usual spacing, C-X_6-C-X_4-C-X_9-C-X_6-C-C (Selsted *et al.*, 1993). The novel genes are classified into four groups: known, related and predicted genes and pseudogenes. ß-defensin genes are classified as known if evidence exists that they are transcribed and that their protein product demonstrates anti-microbial activity. ß-defensin genes are classified as related if evidence yet exists that they are transcribed but their protein product has not been tested for anti-microbial activity. ß-defensin genes are classified as predicted if no evidence exists that they are transcribed, and they are classified as pseudogenes if the DNA sequence is highly similar to a ß-defensin gene, but the predicted amino acid sequence lacks an open reading frame across the six-

cysteine motif. The sequences were aligned as described in Methods followed by minor eye adjustments to maximize sequence alignment and clustering of genes by chromosome. The consensus sequence shows specific residues and residues with the same functional group if they are represented in greater than 30% of all predicted ß-defensin proteins. The cysteines of the six-cysteine motif are in bold face type.

FIG. 2: Dendogram of predicted ß-defensin proteins. The length of each branch is inversely related to their similarity. The tree was constructed with the predicted amino acid sequences derived from the indicated human (closed circle) and mouse (open circle) known, related and predicted genes (Appendix 1). The genomic location for each gene is indicated. In some cases the location of the genes was ambiguous (A) or unkown (U).

FIG. 3: Order and orientation of genes in three of the ß-defensin gene clusters in the human and mouse genomes. The horizontal bars represent the assembled genomic DNA sequence contigs (see Appendix 1 for Genbank and Celera accession numbers for each contig) from the indicated human (Hs) and mouse (Mm) chromosome. Double slanted lines represent gaps in the genomic DNA sequence. The telomere (Tel) and centromere (Cen) orientation of the human DNA sequence contigs was deduced from the position of genetic markers within them. The orientation of the mouse DNA sequence contigs was deduced from the most parsimonious alignment of human and mouse gene homologs. The direction of transcription is indicated for known and related genes (filled arrows) and predicted genes and pseudogenes (open arrows). Thin lines connecting human and mouse genes indicate genes with highest sequence similarity (FIG. 2).

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

The present invention utilizes BLAST and *hmmsearch* tools to expand the ß-defensin gene family from six members in the human genome and six members in the mouse genome to 34 and 48, respectively. While these two sequence analysis tools are not new, the iterative search process used is novel as was the application of the tools to the five frame translations of the draft sequence of the human genome. The BLAST sequence similarity search identified a genomic region that contained at least one hypothetical ß-defensin gene. Subsequently, *hmmsearch* analysis of this region identified additional hypothetical ß-defensin genes that were missed by the BLAST search. The novel sequences were then used as probes in additional BLAST searches and were reseeded into a new HMM. While each new HMM was more

sensitive, the *hmmsearch* tool detected less than half of the sequences used to build the HMM in a genome-wide screen. This method highlights the complementary nature of these two sequence analysis tools and demonstrates their potential synergy for mining genomic databases and identifying new members of gene families.

Pathogenic microbial strains increasingly exhibit resistance or decreased sensitivity to commercially available antibiotics and antimicrobials. While microbial strains have acquired resistance to many commercial antibiotics within a few decades, it does not appear that similar resistance to antimicrobial peptides has been acquired, despite thousands of years of evolution. The antimicrobial properties of β-defensins are thus useful, alone and in combination with other antimicrobial agents, in the inhibition of microbial growth and/or infection.

A person of ordinary skill would recognize that the antimicrobial properties of β-defensin peptides may be exploited in a variety of applications. While preferred embodiments of the invention encompass administration of peptides to a host for therapeutic or prophylactic benefit, it also is envisioned that the peptides will have other uses. In alternate embodiments, it is envisioned that β-defensins may be included in antiseptic or antimicrobial preparations for application or introduction into environments in which an individual wishes to prevent or suppress microbial growth. Thus, for example, in one aspect of the instant invention, β-defensins are diluted in a composition for application to a surface, such as a work surface or a surgical instrument, for the prevention and/or suppression of microbial growth.

Where the antimicrobial peptide is to be provided to a host, the nature of the peptides facilitates a number of alternate routes of administration. The durability of the peptides facilitates not only internal administration but also application of β-defensins in a topical formulation. Where β-defensins are to be given internally, a variety of means of delivery are possible. In a particular embodiment of the invention, the peptides are diluted in a suitable pharmaceutical composition for delivery by inhalation for the treatment or prevention of pulmonary infections. It is further contemplated that the nucleic acid sequence of the peptides may be delivered to cells by an appropriate vector or DNA delivery vehicle in the context of gene therapy.

As antimicrobial peptides have been determined to be important components of the innate immune system, it is envisioned that monitoring expression of the protein *in vivo* may prove to be important in not only detecting latent infection but also potentially as an indicator of immune dysfunction. In each context, β-defensin nucleic acid signal or peptide expression may be monitored by means readily known in the art.

A. β-defensins

β-defensins are cationic peptides with broad-spectrum antimicrobial activity that are products of epithelia and leukocytes (Ganz and Weiss, 1997). These two exon, single gene products are expressed at epithelial surfaces and secreted at sites including the skin (Harder *et al.*, 1997), cornea (McNamara *et al.*, 1999), tongue (Mathews *et al.*, 1999, Jia *et al.*, 2000), gingiva (Mathews *et al.*, 1999; Krisanaprakornkit *et al.*, 1998), salivary glands (Mathews *et al.*, 1999), esophagus (Jia *et al.*, 2000), intestine (O'Neil *et al.*, 1999), kidney (Valore *et al.*, 1998; Zucht *et al.*, 1998), urogenital tract (Valore *et al.*, 1998), and the respiratory epithelium (Bals *et al.*, 1998; Goldman *et al.*, 1997; McCray and Bentley. 1997). To date, five β-defensin genes of epithelial origin, DEFB1 (Bensch *et al.*, 1995), DEFB2 (Harder *et al.*, 1997), DEFB3 (Harder *et al.*, 2001; Jia *et al.*, 2001), DEFB4 and HE2/EP2 have been identified and characterized in humans.

The primary structure of each β-defensin gene product is characterized by small size, a six cysteine motif, high cationic charge and exquisite diversity beyond these features. The most characteristic feature of defensin proteins is their six-cysteine motif that forms a network of three disulfide bonds. The three disulfide bonds in the β-defensin proteins are between C1-C5, C2-C4 and C3-C6. The most common spacing between adjacent cysteine residues is 6, 4, 9, 6, 0. The spacing between the cysteines in the β-defensin proteins can vary by one or two amino acids except for C5 and C6, located nearest the carboxy terminus. In all known vertebrate β-defensin genes, these two cysteine residues are adjacent to each other.

A second feature of the β-defensin proteins is their small size. Each β-defensin gene encodes a preproprotein that ranges in size from 59 to 80 amino acids with an average size of 65 amino acids. This gene product is then cleaved by an unknown mechanism to create the mature peptide that ranges in size from 36 to 47 amino acids with an average size of 45 amino acids. The exceptions to these ranges are the EP2/HE2 gene products that contain the β-defensin motif and are expressed in the epididymis (Frohlich *et al*, 2000; Kirchhoff *et al.*, 1990; Krull *et al.*, 1993; Osterhoff *et al.*, 1994; Frohlich *et al.*, 2001; Hamil *et al.*, 2000). Using alternative splicing and a secondary promoter, the human HE2/EP2 gene produces three isoforms that carry the β-defensin motif EP2C, EP2D and EP2E (Frohlich *et al.*, 2001). The size of the preproproteins is 113, 133 and 80, respectively.

A third feature of β-defensin proteins is the high concentration of cationic residues. The number of positively charged residues (arginine, lysine, histidine) in the mature peptide ranges from 6 to 14 with an average of 9 (Table 2). It has been proposed that the high positive charge density allows the β-defensin peptides to bind and insert into the cellular membrane, where they

kill the cell either by forming a pore (White *et al.*, 1995) or by simply permeablizing the cell through an electrostatic interaction without forming a pore (Hoover *et al.*, 2000). The relationship between the killing activity and the charge density of the β-defensin proteins is supported by the observations that the antimicrobial activity of many β-defensin proteins is salt-sensitive (Valore *et al.*, 1998; Bals *et al.*, 1998; Goldman *et al.*, 1997; Bals and Goldman *et al.*, 1998; Singh *et al.*, 1998; Morrison *et al.*, 1998; Bals *et al.*; 1999; Shi *et al.*, 1999) , possibly by interfering with the binding to the negatively charged bacterial surface. An exception to this rule is the protein encoded by DEFB3 whose bactericidal activity against *Staphylococcus aureus* is not salt-sensitive at physiological salt concentrations (Harder *et al.*, 2001). As noted previously (Jia *et al.*, 2001), the DEFB3 gene encodes six more positively charged amino acids than the other two human β-defensin genes, DEFB1 and DEFB2. Future experiments will likely test whether these additional positive charges are related to the salt-insensitive *Staph* killing activity of this protein.

The final feature of the β-defensin gene products is their diverse primary structure but apparent conservation of tertiary structure. Beyond the six cysteines, no single amino acid at a given position is conserved in all known members of this protein family. However, there are positions that are conserved that appear to be important for secondary and tertiary structures and function (see below).

Despite the great diversity of the primary amino acid sequence of the β-defensin proteins, the limited data suggests that the tertiary structure of this protein family is conserved and provides a unifying theme for antimicrobial activity. The solution structure has been determined for the proteins encoded by BNBD-12 from cow (Zimmermann *et al.*, 1995), DEFB2 from human (Sawai *et al.*, 2001) and DPL1 from platypus (Torres *et al.*, 1999). The structural core for each of these proteins is a triple-stranded, antiparallel β-sheet, as exemplified for the proteins encoded by BNBD-12 and DEFB2. The three β-strands are connected by a β-turn, and a β-hairpin loop, and the second β-strand also contains a β-bulge. When these structures are folded into their proper tertiary structure, the apparently random sequence of cationic and hydrophobic residues are concentrated into two faces of a globular protein. One face is hydrophilic and contains many of the positively charged side chains and the other is hydrophobic. In solution, the HBD-2 protein encoded by the DEFB2 gene exhibited a α-helical segment near the N-terminus not previously ascribed to solution structures of α-defensins or to the ß-defensin BNBD-12. The authors speculated that this novel structural element might contribute to the specific microbicidal or chemokine-like properties of HBD-2 (Sawai *et al.*, 2001). Presumably, it is this amphipathic nature of these proteins that allows them to be effective antimicrobial

agents. As noted above, an electrostatic interaction occurs between the cationic surface of the defensin protein and the polyanionic surface of the bacterial membrane. Then, the hydrophobic surface invades the membrane and ultimately leads to disruption of the membrane. The amino acids whose side chains are directed toward the surface of the protein are less conserved between β-defensin proteins and may partly explain the difference in specificity for antimicrobial activity, while the amino acid residues in the three β-strands of the core β-sheet are more highly conserved.

The most widely studied aspect of ß-defensin function is their antimicrobial properties (Ganz *et al.*, 1999). ß-defensin peptides are produced as pre-pro-peptides and then cleaved to release a C-terminal active peptide fragment, however the pathways for the intracellular processing, storage and release of the human ß-defensin peptides in airway epithelia are unknown. While it is well-documented that ß-defensin peptides are present in mucosal secretions, it is also possible that important antibacterial functions of the peptides may be related to their presence intracellularly or when attached to cell surfaces or secreted mucins. In general, ß-defensin activity is microbicidal rather than bacteriostatic and requires micromolar concentrations. Broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi and enveloped viruses has been reported, but most studies have focused on the antibacterial activity (Ganz *et al.*, 1999; Daher *et al.*, 1986). Characteristically, the antimicrobial activity of the ß-defensin peptides is salt sensitive and their killing is markedly reduced as the ionic strength of the solutions increases (i.e., [NaCl] > 50 mM). However, a striking feature of the HBD-3 peptide is its greater density of cationic residues when compared with HBD-1 and HBD-2, especially at its C-terminus (Harder *et al.*, 2001; Jia *et al.*, 2001). Perhaps the greater charge density of HBD-3 facilitates greater interactions and therefore activity with the Gram-positive bacteria cell wall. Thus from ongoing investigations of the human ß-defensins, patterns of unique antimicrobial spectrums are beginning to emerge. It will be interesting to learn how the variable spectrums of activity may relate to different pathways for inducing peptide expression in response to infection or inflammation.

The defensin peptides are thought to initiate their interactions with bacteria through simple electrostatic interactions with bacterial cell walls. It is this property that confers the characteristic salt-sensitivity to the peptides. Their amphiphilic design allows the peptides to interact with membranes such that the charged regions bind to anionic phospholipid head groups (*i.e.*, LPS, techoic acid) and water and the nonpolar surface is buried in the lipid phase. While there is compelling evidence that defensins permeabilize bacterial cell membranes, the mechanism of the effect is not known. It is well documented that α-defensins sequentially

permeabilize the outer and inner membranes of *E. coli* (Lehrer *et al.*, 1989). Defensins may act by forming oligomeric membrane spanning pores, by disrupting lipid membranes, or through a combination of such effects (Ganz *et al.*, 1999; Sawai *et al.*, 2001; Hoover *et al.*, 2000). The lower anionic lipid content of the cell membranes of multicellular organisms is thought to provide a degree of specificity and protection against damage to host cells.

In addition to their broad spectrum antimicrobial properties, there is evidence that the β-defensins may act as chemokines for immature dendritic cells and memory T cells, and thus serve as a bridge between the innate and adaptive immune systems (Yang *et al.*, 1999; Ganz, 1999). Studies by Yang and colleagues revealed that HBD-1 and HBD-2 were selectively chemotactic for cells expressing the human CCR6, a chemokine receptor preferentially expressed by immature dendritic cells and memory T cells (Yang *et al.*, 1999; Liao *et al.*, 1999; Baba *et al.*, 1997). In contrast to the micromolar concentrations needed to kill bacteria, the ß-defensin chemokine activities were present at nanomolar concentrations (Yang *et al.*, 1999). The HBD-1, -2-induced chemotaxis was sensitive to pertussis toxin and was inhibited by antibodies to CCR6. The binding of iodinated CCL20 (also termed LARC or MIP-3α), the only reported chemokine ligand for CCR6, to CCR6-transfected cells was competitively inhibited by the ß-defensins. The chemokine activity of CCL20 was approximately 10-fold greater than that of HBD-1 and HBD-2 (Yang *et al.*, 1999). Thus, ß-defensins may also promote adaptive immune responses by recruiting dendritic and T cells to the site of microbial invasion through interaction with CCR6. HBD-2 also stimulates mast cells to release histamine (Niyonsaba *et al.*, 2001).

B. Nucleic Acids

The instant invention also relates to genetic sequences for specific genes expressed by immune cells and exhibiting antimicrobial activity. Therefore, the use, manipulation, detection, isolation, amplification and screening of nucleic acids are important aspects of the invention.

In the context of the instant invention, genes are sequences of DNA in an organism's genome encoding information that is converted into various products making up a whole cell. They are expressed by the process of transcription, which involves copying the sequence of DNA into RNA. Most genes encode information to make proteins, but some encode RNAs involved in other processes. If a gene encodes a protein, its transcription product is called mRNA ("messenger" RNA). After transcription in the nucleus (where DNA is located), the mRNA must be transported into the cytoplasm for the process of translation, which converts the code of the mRNA into a sequence of amino acids to form protein. In order to direct transport into the cytoplasm, the 3′ ends of mRNA molecules are post-transcriptionally modified by addition of several adenylate residues to form the "polyA" tail. This characteristic modification

distinguishes gene expression products destined to make protein from other molecules in the cell, and thereby provides one means for detecting and monitoring the gene expression activities of a cell.

The term "nucleic acid" will generally refer to at least one molecule or strand of DNA, RNA or a derivative or mimic thereof, comprising at least one nucleobase, such as, for example, a naturally occurring purine or pyrimidine base found in DNA (*e.g.*, adenine "A," guanine "G," thymine "T" and cytosine "C") or RNA (*e.g.*, A, G, uracil "U" and C). The term "nucleic acid" encompass the terms "oligonucleotide" and "polynucleotide." The term "oligonucleotide" refers to at least one molecule of between about 3 and about 100 nucleobases in length. The term "polynucleotide" refers to at least one molecule of greater than about 100 nucleobases in length. These definitions generally refer to at least one single-stranded molecule, but in specific embodiments will also encompass at least one additional strand that is partially, substantially or fully complementary to the at least one single-stranded molecule. Thus, a nucleic acid may encompass at least one double-stranded molecule or at least one triple-stranded molecule that comprises one or more complementary strand(s) or "complement(s)" of a particular sequence comprising a strand of the molecule. As used herein, a single stranded nucleic acid may be denoted by the prefix "ss", a double stranded nucleic acid by the prefix "ds", and a triple stranded nucleic acid by the prefix "ts."

Nucleic acid(s) that are "complementary" or "complement(s)" are those that are capable of base-pairing according to the standard Watson-Crick, Hoogsteen or reverse Hoogsteen binding complementarity rules. As used herein, the term "complementary" or "complement(s)" also refers to nucleic acid(s) that are substantially complementary, as may be assessed by the same nucleotide comparison set forth above. The term "substantially complementary" refers to a nucleic acid comprising at least one sequence of consecutive nucleobases, or semiconsecutive nucleobases if one or more nucleobase moieties are not present in the molecule, are capable of hybridizing to at least one nucleic acid strand or duplex even if less than all nucleobases do not base pair with a counterpart nucleobase. In certain embodiments, a "substantially complementary" nucleic acid contains at least one sequence in which about 70%, about 71%, about 72%, about 73%, about 74%, about 75%, about 76%, about 77%, about 77%, about 78%, about 79%, about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, to about 100%, and any range therein, of the nucleobase sequence is capable of base-pairing with at least one single or double stranded nucleic acid molecule during hybridization. In certain embodiments, the term

"substantially complementary" refers to at least one nucleic acid that may hybridize to at least one nucleic acid strand or duplex in stringent conditions. In certain embodiments, a "partly complementary" nucleic acid comprises at least one sequence that may hybridize in low stringency conditions to at least one single or double stranded nucleic acid, or contains at least one sequence in which less than about 70% of the nucleobase sequence is capable of base-pairing with at least one single or double stranded nucleic acid molecule during hybridization.

Hybridization is understood to mean the forming of a double stranded molecule and/or a molecule with partial double stranded nature. Stringent conditions are those that allow hybridization between two homologous nucleic acid sequences, but precludes hybridization of random sequences. For example, hybridization at low temperature and/or high ionic strength is termed low stringency. Hybridization at high temperature and/or low ionic strength is termed high stringency. Low stringency is generally performed at 0.15 M to 0.9 M NaCl at a temperature range of 20°C to 50°C. High stringency is generally performed at 0.02 M to 0.15 M NaCl at a temperature range of 50°C to 70°C. It is understood that the temperature and/or ionic strength of a desired stringency are determined in part by the length of the particular probe, the length and/or base content of the target sequences, and/or to the presence of formamide, tetramethylammonium chloride and/or other solvents in the hybridization mixture. It is also understood that these ranges are mentioned by way of example only, and/or that the desired stringency for a particular hybridization reaction is often determined empirically by comparison to positive and/or negative controls.

Accordingly, the nucleotide sequences of the disclosure may be used for their ability to selectively form duplex molecules with complementary stretches of genes and/or RNA. Depending on the application envisioned, it is preferred to employ varying conditions of hybridization to achieve varying degrees of selectivity of probe towards target sequence.

Nucleic acid molecules having sequence regions consisting of contiguous nucleotide stretches of about 13, 14, 15, 16, 17, 18, 20, 25, 30, 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 125, 150, 175, 200, or 250 identical or complementary to the target DNA sequence, are particularly contemplated as hybridization probes for use in embodiments of the instant invention. It is contemplated that long contiguous sequence regions, for use in, for example, genomic screening, may be utilized including those sequences comprising about 100, 200, 300, 400, 500 or more contiguous nucleotides.

The use of a probe or primer of between 13 and 100 nucleotides, preferably between 17 and 100 nucleotides in length, or in some aspects of the invention up to 1-2 kb or more in length, allows the formation of a duplex molecule that is both stable and selective. Molecules having

complementary sequences over contiguous stretches greater than 20 bases in length are generally preferred, to increase stability and/or selectivity of the hybrid molecules obtained. One will generally prefer to design nucleic acid molecules for hybridization having one or more complementary sequences of 20 to 30 nucleotides, or even longer where desired. Such fragments may be readily prepared, for example, by directly synthesizing the fragment by chemical means or by introducing selected sequences into recombinant vectors for recombinant production.

Depending on the application envisioned, one would desire to employ varying conditions of hybridization to achieve varying degrees of selectivity of the probe or primers for the target sequence. For applications requiring high selectivity, one will typically desire to employ relatively high stringency conditions to form the hybrids. For example, relatively low salt and/or high temperature conditions, such as provided by about 0.02 M to about 0.10 M NaCl at temperatures of about 50°C to about 70°C. Such high stringency conditions tolerate little, if any, mismatch between the probe or primers and the template or target strand and would be particularly suitable for isolating specific genes or for detecting specific mRNA transcripts. It is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide.

Conditions may be rendered less stringent by increasing salt concentration and/or decreasing temperature. For example, a medium stringency condition could be provided by about 0.1 to 0.25 M NaCl at temperatures of about 37°C to about 55°C, while a low stringency condition could be provided by about 0.15 M to about 0.9 M salt, at temperatures ranging from about 20°C to about 55°C. Hybridization conditions can be readily manipulated depending on the desired results.

In other embodiments, hybridization may be achieved under conditions of, for example, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 1.0 mM dithiothreitol, at temperatures between approximately 20°C to about 37°C. Other hybridization conditions utilized could include approximately 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, at temperatures ranging from approximately 40°C to about 72°C.

As used herein "stringent condition(s)" or "high stringency" are those that allow hybridization between or within one or more nucleic acid strand(s) containing complementary sequence(s), but precludes hybridization of random sequences. Stringent conditions tolerate little, if any, mismatch between a nucleic acid and a target strand. Such conditions are well known to those of ordinary skill in the art, and are preferred for applications requiring high selectivity. Non-limiting applications include isolating at least one nucleic acid, such as a gene or nucleic acid segment thereof, or detecting at least one specific mRNA transcript or nucleic acid segment thereof, and the like.

For applications requiring high selectivity, it is preferred to employ relatively stringent conditions to form the hybrids. For example, relatively low salt and/or high temperature conditions, such as provided by about 0.02 M to about 0.10 M NaCl at temperatures of about 50°C to about 70°C. Such high stringency conditions tolerate little, if any, mismatch between the probe and/or the template and/or target strand, and/or would be particularly suitable for isolating specific genes and/or detecting specific mRNA transcripts. It is generally appreciated that conditions may be rendered more stringent by the addition of increasing amounts of formamide.

In the context of the instant application, nucleic acids are also important for expression systems producing the claimed peptide.

C. Peptide Production

A person of ordinary skill would be aware of a variety of means of producing, isolating, purifying and manipulating the peptide molecules set forth herein. Exemplary methods are briefly summarized below.

1. Peptide Synthesis

a. Chemical Synthesis

The antimicrobial peptides of the instant invention may be chemically synthesized. An exemplary method for chemical synthesis of such a peptide is as follows. Using the solid phase peptide synthesis method of Sheppard *et al.* (1981) an automated peptide synthesizer (Pharmacia LKB Biotechnology Co., LKB Biotynk 4170) adds N,N'-dicyclohexylcarbodiimide to amino acids whose amine functional groups are protected by 9-fluorenylmethoxycarbonyl groups, producing anhydrides of the desired amino acid (Fmoc-amino acids). An Fmoc amino acid corresponding to the C-terminal amino acid of the desired peptide is affixed to Ultrosyn A resin (Pharmacia LKB Biotechnology Co.) through its carboxyl group, using dimethylaminopyridine as a catalyst. The resin is then washed with dimethylformamide containing iperidine resulting in the removal of the protective amine group of the C-terminal amino acid. A Fmoc-amino acid anhydride corresponding to the next residue in the peptide sequence is then added to the substrate and allowed to couple with the unprotected amino acid affixed to the resin. The protective amine group is subsequently removed from the second amino acid and the above process is repeated with additional residues added to the peptide in a like manner until the sequence is completed. After the peptide is completed, the protective groups, other than the acetoamidomethyl group are removed and the peptide is released from the resin with a solvent consisting of, for example, 94% (by weight) trifluoroacetic acid, 5% phenol, and 1% ethanol. The synthesized peptide is subsequently purified using high-performance liquid chromatography

or other peptide purification techniques discussed below and must then be oxidized to properly form three disulfide bonds.

b. Expression Systems

The antimicrobial peptides of the instant invention may be expressed by a prokaryotic or eukaryotic expression vector. The term "expression vector" refers to a vector containing a nucleic acid sequence coding for at least part of a gene product capable of being transcribed. In some cases, RNA molecules are then translated into a protein, polypeptide, or peptide. In other cases, these sequences are not translated, for example, in the production of antisense molecules or ribozymes. Expression vectors can contain a variety of "control sequences," which refer to nucleic acid sequences necessary for the transcription and possibly translation of an operably linked coding sequence in a particular host organism. In addition to control sequences that govern transcription and translation, vectors and expression vectors may contain nucleic acid sequences that serve other functions as well and are described infra.

1. Promoters and Enhancers

A "promoter" is a control sequence that is a region of a nucleic acid sequence at which initiation and rate of transcription are controlled. It may contain genetic elements at which regulatory proteins and molecules may bind such as RNA polymerase and other transcription factors. The phrases "operatively positioned," "operatively linked," "under control," and "under transcriptional control" mean that a promoter is in a correct functional location and/or orientation in relation to a nucleic acid sequence to control transcriptional initiation and/or expression of that sequence. A promoter may or may not be used in conjunction with an "enhancer," which refers to a cis-acting regulatory sequence involved in the transcriptional activation of a nucleic acid sequence.

A promoter may be one naturally associated with a gene or sequence, as may be obtained by isolating the 5′ non-coding sequences located upstream of the coding segment and/or exon. Such a promoter can be referred to as "endogenous." Similarly, an enhancer may be one naturally associated with a nucleic acid sequence, located either downstream or upstream of that sequence. Alternatively, certain advantages will be gained by positioning the coding nucleic acid segment under the control of a recombinant or heterologous promoter, which refers to a promoter that is not normally associated with a nucleic acid sequence in its natural environment. A recombinant or heterologous enhancer refers also to an enhancer not normally associated with a nucleic acid sequence in its natural environment. Such promoters or enhancers may include promoters or enhancers of other genes, and promoters or enhancers isolated from any other

prokaryotic, viral, or eukaryotic cell, and promoters or enhancers not "naturally occurring," *i.e.*, containing different elements of different transcriptional regulatory regions, and/or mutations that alter expression. In addition to producing nucleic acid sequences of promoters and enhancers synthetically, sequences may be produced using recombinant cloning and/or nucleic acid amplification technology, including PCR™, in connection with the compositions disclosed herein (see U.S. Patent 4,683,202; U.S. Patent 5,928,906, each incorporated herein by reference). Furthermore, it is contemplated the control sequences that direct transcription and/or expression of sequences within non-nuclear organelles such as mitochondria, chloroplasts, and the like, can be employed as well.

Naturally, it will be important to employ a promoter and/or enhancer that effectively directs the expression of the DNA segment in the cell type, organelle, and organism chosen for expression. Those of skill in the art of molecular biology generally know the use of promoters, enhancers, and cell type combinations for protein expression, for example, see Sambrook *et al.* (1989), incorporated herein by reference. The promoters employed may be constitutive, tissue-specific, inducible, and/or useful under the appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins and/or peptides. The promoter may be heterologous or endogenous.

Table 1 lists several elements/promoters that may be employed, in the context of the present invention, to regulate the expression of a gene. This list is not intended to be exhaustive of all the possible elements involved in the promotion of expression but, merely, to be exemplary thereof. Table 2 provides examples of inducible elements, which are regions of a nucleic acid sequence that can be activated in response to a specific stimulus.

TABLE 1

Promoter and/or Enhancer

Promoter/Enhancer	References
Immunoglobulin Heavy Chain	Banerji *et al.*, 1983; Gilles *et al.*, 1983; Grosschedl *et al.*, 1985; Imler *et al.*, 1987; Weinberger *et al.*, 1984; Kiledjian *et al.*, 1988; Porton *et al.*; 1990
Immunoglobulin Light Chain	Queen *et al.*, 1983; Picard *et al.*, 1984
T-Cell Receptor	Luria *et al.*, 1987; Winoto *et al.*, 1989; Redondo *et al.*; 1990
HLA DQ a and/or DQ β	Sullivan *et al.*, 1987
β-Interferon	Goodbourn *et al.*, 1986; Fujita *et al.*, 1987; Goodbourn *et al.*, 1988

TABLE 1

Promoter and/or Enhancer

Promoter/Enhancer	References
Interleukin-2	Greene *et al.*, 1989
Interleukin-2 Receptor	Greene *et al.*, 1989; Lin *et al.*, 1990
MHC Class II 5	Koch *et al.*, 1989
MHC Class II HLA-DRa	Sherman *et al.*, 1989
β-Actin	Kawamoto *et al.*, 1988; Ng *et al.*; 1989
Muscle Creatine Kinase (MCK)	Jaynes *et al.*, 1988; Horlick *et al.*, 1989; Johnson *et al.*, 1989
Prealbumin (Transthyretin)	Costa *et al.*, 1988
Elastase I	Omnitz *et al.*, 1987
Metallothionein (MTII)	Karin *et al.*, 1987; Culotta *et al.*, 1989
Collagenase	Pinkert *et al.*, 1987; Angel *et al.*, 1987
Albumin	Pinkert *et al.*, 1987; Tronche *et al.*, 1989, 1990
α-Fetoprotein	Godbout *et al.*, 1988; Campere *et al.*, 1989
t-Globin	Bodine *et al.*, 1987; Perez-Stable *et al.*, 1990
β-Globin	Trudel *et al.*, 1987
c-fos	Cohen *et al.*, 1987
c-HA-*ras*	Treisman, 1986; Deschamps *et al.*, 1985
Insulin	Edlund *et al.*, 1985
Neural Cell Adhesion Molecule (NCAM)	Hirsch *et al.*, 1990
α_1-Antitrypain	Latimer *et al.*, 1990
H2B (TH2B) Histone	Hwang *et al.*, 1990
Mouse and/or Type I Collagen	Ripe *et al.*, 1989
Glucose-Regulated Proteins (GRP94 and GRP78)	Chang *et al.*, 1989
Rat Growth Hormone	Larsen *et al.*, 1986
Human Serum Amyloid A (SAA)	Edbrooke *et al.*, 1989
Troponin I (TN I)	Yutzey *et al.*, 1989
Platelet-Derived Growth Factor (PDGF)	Pech *et al.*, 1989
Duchenne Muscular Dystrophy	Klamut *et al.*, 1990
SV40	Banerji *et al.*, 1981; Moreau *et al.*, 1981; Sleigh *et al.*, 1985; Firak *et al.*, 1986; Herr *et al.*, 1986; Imbra *et al.*, 1986; Kadesch *et al.*, 1986; Wang *et al.*, 1986; Ondek *et al.*, 1987; Kuhl *et al.*, 1987; Schaffner *et al.*, 1988
Polyoma	Swartzendruber *et al.*, 1975; Vasseur *et al.*, 1980; Katinka *et al.*, 1980, 1981; Tyndell *et al.*, 1981; Dandolo *et al.*, 1983; de Villiers *et al.*, 1984; Hen *et al.*, 1986; Satake *et al.*, 1988; Campbell and/or Villarreal, 1988

TABLE 1

Promoter and/or Enhancer

Promoter/Enhancer	References
Retroviruses	Kriegler *et al.*, 1982, 1983; Levinson *et al.*, 1982; Kriegler *et al.*, 1983, 1984a, b, 1988; Bosze *et al.*, 1986; Miksicek *et al.*, 1986; Celander *et al.*, 1987; Thiesen *et al.*, 1988; Celander *et al.*, 1988; Choi *et al.*, 1988; Reisman *et al.*, 1989
Papilloma Virus	Campo *et al.*, 1983; Lusky *et al.*, 1983; Spandidos and/or Wilkie, 1983; Spalholz *et al.*, 1985; Lusky *et al.*, 1986; Cripe *et al.*, 1987; Gloss *et al.*, 1987; Hirochika *et al.*, 1987; Stephens *et al.*, 1987
Hepatitis B Virus	Bulla *et al.*, 1986; Jameel *et al.*, 1986; Shaul *et al.*, 1987; Spandau *et al.*, 1988; Vannice *et al.*, 1988
Human Immunodeficiency Virus	Muesing *et al.*, 1987; Hauber *et al.*, 1988; Jakobovits *et al.*, 1988; Feng *et al.*, 1988; Takebe *et al.*, 1988; Rosen *et al.*, 1988; Berkhout *et al.*, 1989; Laspia *et al.*, 1989; Sharp *et al.*, 1989; Braddock *et al.*, 1989
Cytomegalovirus (CMV)	Weber *et al.*, 1984; Boshart *et al.*, 1985; Foecking *et al.*, 1986
Gibbon Ape Leukemia Virus	Holbrook *et al.*, 1987; Quinn *et al.*, 1989

TABLE 2

Inducible Elements

Element	Inducer	References
MT II	Phorbol Ester (TFA) Heavy metals	Palmiter *et al.*, 1982; Haslinger *et al.*, 1985; Searle *et al.*, 1985; Stuart *et al.*, 1985; Imagawa *et al.*, 1987, Karin *et al.*, 1987; Angel *et al.*, 1987b; McNeall *et al.*, 1989
MMTV (mouse mammary tumor virus)	Glucocorticoids	Huang *et al.*, 1981; Lee *et al.*, 1981; Majors *et al.*, 1983; Chandler *et al.*, 1983; Ponta *et al.*, 1985
β-Interferon	poly(rI)x poly(rc)	Tavernier *et al.*, 1983
Adenovirus 5 E2	EIA	Imperiale *et al.*, 1984
Collagenase	Phorbol Ester (TPA)	Angel *et al.*, 1987a
Stromelysin	Phorbol Ester (TPA)	Angel *et al.*, 1987b

TABLE 2

Inducible Elements

Element	Inducer	References
SV40	Phorbol Ester (TPA)	Angel *et al.*, 1987b
Murine MX Gene	Interferon, Newcastle Disease Virus	Hug *et al.*, 1988
GRP78 Gene	A23187	Resendez *et al.*, 1988
α-2-Macroglobulin	IL-6	Kunz *et al.*, 1989
Vimentin	Serum	Rittling *et al.*, 1989
MHC Class I Gene H-2κb	Interferon	Blanar *et al.*, 1989
HSP70	E1A, SV40 Large T Antigen	Taylor *et al.*, 1989, 1990a, 1990b
Proliferin	Phorbol Ester-TPA	Mordacq *et al.*, 1989
Tumor Necrosis Factor	PMA	Hensel *et al.*, 1989
Thyroid Stimulating Hormone α Gene	Thyroid Hormone	Chatterjee *et al.*, 1989

The identity of tissue-specific promoters or elements, as well as assays to characterize their activity, is well known to those of skill in the art. Examples of such regions include the human LIMK2 gene (Nomoto *et al.* 1999), the somatostatin receptor 2 gene (Kraus *et al.*, 1998), murine epididymal retinoic acid-binding gene (Lareyre *et al.*, 1999), human CD4 (Zhao-Emonet *et al.*, 1998), mouse alpha 2 (XI) collagen (Tsumaki *et al.*, 1998), D1A dopamine receptor gene (Lee *et al.*, 1997), insulin-like growth factor II (Wu *et al.*, 1997), human platelet endothelial cell adhesion molecule-1 (Almendro *et al.*, 1996).

2. Initiation Signals and Internal Ribosome Binding Sites

A specific initiation signal also may be required for efficient translation of coding sequences. These signals include the ATG initiation codon or adjacent sequences. Exogenous translational control signals, including the ATG initiation codon, may need to be provided. One of ordinary skill in the art would readily be capable of determining this and providing the necessary signals. It is well known that the initiation codon must be "in-frame" with the reading frame of the desired coding sequence to ensure translation of the entire insert. The exogenous translational control signals and initiation codons can be either natural or synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements.

In certain embodiments of the invention, the use of internal ribosome entry sites (IRES) elements are used to create multigene, or polycistronic, messages. IRES elements are able to

bypass the ribosome scanning model of methylated Cap dependent translation and begin translation at internal sites (Pelletier and Sonenberg, 1988). IRES elements from two members of the picornavirus family (polio and encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988), as well an IRES from a mammalian message (Macejak and Sarnow, 1991). IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message. See also U.S. Patents 5,925,565 and 5,935,819, herein incorporated by reference.

3. Multiple Cloning Sites

Vectors can include a multiple cloning site (MCS), which is a nucleic acid region that contains multiple restriction enzyme sites, any of which can be used in conjunction with standard recombinant technology to digest the vector. See Carbonelli *et al.*, 1999; Levenson *et al.*; 1998, and Cocea, 1997; incorporated herein by reference. "Restriction enzyme digestion" refers to catalytic cleavage of a nucleic acid molecule with an enzyme that functions only at specific locations in a nucleic acid molecule. Many of these restriction enzymes are commercially available. Use of such enzymes is widely understood by those of skill in the art. Frequently, a vector is linearized or fragmented using a restriction enzyme that cuts within the MCS to enable exogenous sequences to be ligated to the vector. "Ligation" refers to the process of forming phosphodiester bonds between two nucleic acid fragments, which may or may not be contiguous with each other. Techniques involving restriction enzymes and ligation reactions are well known to those of skill in the art of recombinant technology.

4. Splicing Sites

Most transcribed eukaryotic RNA molecules will undergo RNA splicing to remove introns from the primary transcripts. Vectors containing genomic eukaryotic sequences may require donor and/or acceptor splicing sites to ensure proper processing of the transcript for protein expression. See Chandler *et al.*, 1997, herein incorporated by reference.

5. Termination Signals

The vectors or constructs of the present invention will generally comprise at least one termination signal. A "termination signal" or "terminator" is comprised of the DNA sequences involved in specific termination of an RNA transcript by an RNA polymerase. Thus, in certain

embodiments a termination signal that ends the production of an RNA transcript is contemplated. A terminator may be necessary in vivo to achieve desirable message levels.

In eukaryotic systems, the terminator region may also comprise specific DNA sequences that permit site-specific cleavage of the new transcript so as to expose a polyadenylation site. This signals a specialized endogenous polymerase to add a stretch of about 200 A residues (polyA) to the 3' end of the transcript. RNA molecules modified with this polyA tail appear to more stable and are translated more efficiently. Thus, in other embodiments involving eukaryotes, it is preferred that that terminator comprises a signal for the cleavage of the RNA, and it is more preferred that the terminator signal promotes polyadenylation of the message. The terminator and/or polyadenylation site elements can serve to enhance message levels and/or to minimize read through from the cassette into other sequences.

Terminators contemplated for use in the invention include any known terminator of transcription described herein or known to one of ordinary skill in the art, including but not limited to, for example, the termination sequences of genes, such as for example the bovine growth hormone terminator or viral termination sequences, such as for example the SV40 terminator. In certain embodiments, the termination signal may be a lack of transcribable or translatable sequence, such as due to a sequence truncation.

6. Polyadenylation Signals

In expression, particularly eukaryotic expression, one will typically include a polyadenylation signal to effect proper polyadenylation of the transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and/or any such sequence may be employed. Preferred embodiments include the SV40 polyadenylation signal and/or the bovine growth hormone polyadenylation signal, convenient and/or known to function well in various target cells. Polyadenylation may increase the stability of the transcript or may facilitate cytoplasmic transport.

7. Origins of Replication

In order to propagate a vector in a host cell, it may contain one or more origins of replication sites (often termed "ori"), which is a specific nucleic acid sequence at which replication is initiated. Alternatively an autonomously replicating sequence (ARS) can be employed if the host cell is yeast.

8. Selectable and Screenable Markers

In certain embodiments of the invention, cells containing a nucleic acid construct of the present invention may be identified in vitro or in vivo by including a marker in the expression

vector. Such markers would confer an identifiable change to the cell permitting easy identification of cells containing the expression vector. Generally, a selectable marker is one that confers a property that allows for selection. A positive selectable marker is one in which the presence of the marker allows for its selection, while a negative selectable marker is one in which its presence prevents its selection. An example of a positive selectable marker is a drug resistance marker.

Usually the inclusion of a drug selection marker aids in the cloning and identification of transformants, for example, genes that confer resistance to neomycin, puromycin, hygromycin, DHFR, GPT, zeocin and histidinol are useful selectable markers. In addition to markers conferring a phenotype that allows for the discrimination of transformants based on the implementation of conditions, other types of markers including screenable markers such as GFP, whose basis is colorimetric analysis, are also contemplated. Alternatively, screenable enzymes such as herpes simplex virus thymidine kinase (tk) or chloramphenicol acetyltransferase (CAT) may be utilized. One of skill in the art would also know how to employ immunologic markers, possibly in conjunction with FACS analysis. The marker used is not believed to be important, so long as it is capable of being expressed simultaneously with the nucleic acid encoding a gene product. Further examples of selectable and screenable markers are well known to one of skill in the art.

9. Host Cells

As used herein, the terms "cell," "cell line," and "cell culture" may be used interchangeably. All of these terms also include their progeny, which is any and all subsequent generations. It is understood that all progeny may not be identical due to deliberate or inadvertent mutations. In the context of expressing a heterologous nucleic acid sequence, "host cell" refers to a prokaryotic or eukaryotic cell, and it includes any transformable organisms that is capable of replicating a vector and/or expressing a heterologous gene encoded by a vector. A host cell can, and has been, used as a recipient for vectors. A host cell may be "transfected" or "transformed," which refers to a process by which exogenous nucleic acid is transferred or introduced into the host cell. A transformed cell includes the primary subject cell and its progeny.

Host cells may be derived from prokaryotes or eukaryotes, depending upon whether the desired result is replication of the vector or expression of part or all of the vector-encoded nucleic acid sequences. Numerous cell lines and cultures are available for use as a host cell, and they can be obtained through the American Type Culture Collection (ATCC), which is an organization that serves as an archive for living cultures and genetic materials. An appropriate

host can be determined by one of skill in the art based on the vector backbone and the desired result. A plasmid or cosmid, for example, can be introduced into a prokaryote host cell for replication of many vectors. Bacterial cells used as host cells for vector replication and/or expression include DH5α, JM109, and KC8, as well as a number of commercially available bacterial hosts such as SURE® Competent Cells and Solopack™ Gold Cells (Stratagene®, La Jolla). Alternatively, bacterial cells such as *E. coli* LE392 could be used as host cells for phage viruses.

Examples of eukaryotic host cells for replication and/or expression of a vector include HeLa, NIH3T3, Jurkat, 293, Cos, CHO, Saos, and PC12. Many host cells from various cell types and organisms are available and would be known to one of skill in the art. Similarly, a viral vector may be used in conjunction with either a eukaryotic or prokaryotic host cell, particularly one that is permissive for replication or expression of the vector.

Some vectors may employ control sequences that allow it to be replicated and/or expressed in both prokaryotic and eukaryotic cells. One of skill in the art would further understand the conditions under which to incubate all of the above described host cells to maintain them and to permit replication of a vector. Also understood and known are techniques and conditions that would allow large-scale production of vectors, as well as production of the nucleic acids encoded by vectors and their cognate polypeptides, proteins, or peptides.

10. Expression Systems

Numerous expression systems exist that comprise at least a part or all of the compositions discussed above. Prokaryote- and/or eukaryote-based systems can be employed for use with the present invention to produce nucleic acid sequences, or their cognate polypeptides, proteins and peptides. Many such systems are commercially and widely available.

The insect cell/baculovirus system can produce a high level of protein expression of a heterologous nucleic acid segment, such as described in U.S. Patents 5,871,986 and 4,879,236, both herein incorporated by reference, and which can be bought, for example, under the name MaxBac® 2.0 from Invitrogen® and BacPack™ Baculovirus Expression System From Clontech®.

Other examples of expression systems include Stratagene®'s Complete Control™ Inducible Mammalian Expression System, which involves a synthetic ecdysone-inducible receptor, or its pET Expression System, an *E. coli* expression system. Another example of an inducible expression system is available from Invitrogen®, which carries the T-Rex™ (tetracycline-regulated expression) System, an inducible mammalian expression system that uses the full-length CMV promoter. Invitrogen® also provides a yeast expression system called the

Pichia methanolica Expression System, which is designed for high-level production of recombinant proteins in the methylotrophic yeast *Pichia methanolica*. One of skill in the art would know how to express a vector, such as an expression construct, to produce a nucleic acid sequence or its cognate polypeptide, protein, or peptide.

2. Fusion Proteins

The antimicrobial peptides of the instant application may be combined with fusion partners to produce fusion proteins. It is envisioned that such constructs might include combinations of an antimicrobial peptide with a partner also exhibiting some level of antimicrobial activity. Such a construct generally has all or a substantial portion of the native molecule, linked at the N- or C-terminus, to all or a portion of a second polypeptide. For example, fusions typically employ leader sequences from other species to permit the recombinant expression of a protein in a heterologous host. Another useful fusion includes the addition of an immunologically active domain, such as an antibody epitope, to facilitate purification of the fusion protein. Inclusion of a cleavage site at or near the fusion junction will facilitate removal of the extraneous polypeptide after purification if such removal is desired. Other useful fusions include linking of functional domains, such as active sites from enzymes, glycosylation domains, cellular targeting signals or transmembrane regions.

It is envisioned that, to construct fusion proteins, the cDNA sequence encoding the antimicrobial peptide would be linked to the cDNA sequence encoding the desired fusion partner. The antimicrobial peptide sequences disclosed in this application allow for the deduction of encoding DNA. Such sequences may be prepared using conventional techniques, and used as probes to recover corresponding DNA's from genomic or cDNA libraries. Following cloning, such DNA's can then be incorporated in appropriate expression vectors and used to transform host cells (*e.g.*, bacterial or mammalian cells), which can be cultured to form recombinant antimicrobial peptides.

3. Peptide Substitutions

As modifications and changes may be made in the structure of the β-defensin gene and peptides or proteins of the present invention, and still obtain molecules having like or otherwise desirable characteristics, such biologically functional equivalents are also encompassed within the present invention.

It is contemplated that specific modifications may be made within the peptide that maintain the peptides antimicrobial properties of the claimed sequence, but also confers some additional desirable property to the peptide. It is well known in the art that certain amino acids

may be substituted for other amino acids in a protein structure without appreciable loss of peptide activity. Since it is the interactive capacity and nature of a peptide that defines that peptide's biological functional activity, certain amino acid sequence substitutions can be made in a protein sequence (or, of course, its underlying DNA coding sequence) and nevertheless obtain a peptide with like properties. It is thus contemplated by the inventors that various changes may be made in the sequence of β-defensin peptides, or the underlying nucleic acids, without appreciable loss of biological utility or activity and perhaps may enhance desired activities.

For example, in designing peptide constructs with antimicrobial properties, substitutions may be used which modulate one or more properties of the molecule. Such variants typically contain the exchange of one amino acid for another at one or more sites within the peptide. For example, certain amino acids may be substituted for other amino acids in a peptide structure in order to enhance the interactive binding capacity of the structures. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid substitutions can be made in a protein sequence, and its underlying DNA coding sequence which potentially create a peptide with superior characteristics.

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte and Doolittle, 1982). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics (Kyte and Doolittle, 1982), these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, *i.e.*, still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Patent 4,554,101, incorporated herein by

reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein. As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ± 1); alanine (-0.5); histidine -0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

Amino acid substitutions are generally based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like but may nevertheless be made to highlight a particular property of the peptide. Exemplary substitutions that take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

As used in this application, the term "an isolated nucleic acid encoding a antimicrobial peptide refers to a nucleic acid molecule that has been isolated free of total cellular nucleic acid. The term "functionally equivalent codon" is used herein to refer to codons that encode the same amino acid, such as the six codons for arginine or serine (Table 3, below), and also refers to codons that encode biologically equivalent amino acids, as discussed in the following pages.

TABLE 3 CODONS

Amino Acids			Codons					
Alanine	Ala	A	GCA	GCC	GCG	GCU		
Cysteine	Cys	C	UGC	UGU				
Aspartic acid	Asp	D	GAC	GAU				
Glutamic acid	Glu	E	GAA	GAG				
Phenylalanine	Phe	F	UUC	UUU				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Lysine	Lys	K	AAA	AAG				
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	Met	M	AUG					
Asparagine	Asn	N	AAC	AAU				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Glutamine	Gln	Q	CAA	CAG				
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Valine	Val	V	GUA	GUC	GUG	GUU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				

Allowing for the degeneracy of the genetic code, sequences that have at least about 50%, usually at least about 60%, more usually about 70%, most usually about 80%, preferably at least about 90% and most preferably about 95% of nucleotides that are identical to the nucleotides of an antimicrobial peptide gene will be sequences that encompassed by the present invention. Nucleic acid sequences of the present invention may also be functionally defined as sequences that are capable of hybridizing to a nucleic acid segment encoding an antimicrobial peptide.

The DNA segments of the present invention include those encoding biologically functional equivalent antimicrobial peptides, as described above. Functionally equivalent proteins or peptides may be created via the application of recombinant DNA technology, in which changes in the protein structure may be engineered, based on considerations of the properties of the amino acids being exchanged, or as a result of natural selection. Changes designed by man may be introduced through the application of site-directed mutagenesis techniques or may be introduced randomly and screened later for the desired function.

4. Protein Purification

Peptide purification techniques are well known to those of skill in the art. These techniques involve, at one level, the crude fractionation of the cellular milieu to polypeptide and non-polypeptide fractions. Having separated the polypeptide from other proteins, the polypeptide of interest may be further purified using chromatographic, immunologic and electrophoretic techniques to achieve partial or complete purification (or purification to homogeneity). Analytical methods particularly suited to the preparation of a pure peptide are ion-exchange chromatography, exclusion chromatography; polyacrylamide gel electrophoresis; isoelectric focusing. A particularly efficient method of purifying peptides is fast protein liquid chromatography or HPLC.

Certain aspects of the present invention concern the purification, and in particular embodiments, the substantial purification, of an encoded peptide. The term "purified peptide" as used herein, is intended to refer to a composition, isolatable from other components, wherein the peptide is purified to any degree relative to its naturally-obtainable state. A purified peptide therefore also refers to a peptide, free from the environment in which it may naturally occur.

Generally, "purified" will refer to a peptide composition that has been subjected to fractionation to remove various other components, and which composition substantially retains its expressed biological activity. Where the term "substantially purified" is used, this designation will refer to a composition in which the protein or peptide forms the major component of the composition, such as constituting about 50%, about 60%, about 70%, about 80%, about 90%, about 95% or more peptides in the composition. The term "purified to homogeneity" is used to mean that the composition has been purified such that there is single protein species based on the particular test of purity employed for example SDS-PAGE or HPLC.

Various methods for quantifying the degree of purification of the peptide will be known to those of skill in the art in light of the present disclosure. These include, for example, assessing the amount of peptides within a fraction by SDS/PAGE analysis.

There is no general requirement that the peptide always be provided in their most purified state. Indeed, it is contemplated that less substantially purified products will have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed utilizing an HPLC apparatus will generally result in a greater "-fold" purification than the same technique utilizing a low pressure chromatography system. Methods exhibiting a lower degree of relative

purification may have advantages in total recovery of protein product, or in maintaining the activity of an expressed protein.

It is particularly contemplated that the peptides of the instant invention may be isolated, purified or visualized on denaturing and non-denaturing gels, particularly acid urea gels. Generally, cationic peptides such as beta defensins are visualized on acid urea western blots or gels where the proteins migrate according to their charge. Persons of skill in the art would be aware of these and other analogous methods, such as, for example SDS/PAGE. It is known that the migration of a peptide can vary, sometimes significantly, with different conditions of acid urea gels or SDS/PAGE (Capaldi *et al.*, 1977). It will therefore be appreciated that under differing electrophoresis conditions, the apparent molecular weights of purified or partially purified expression products may vary.

High Performance Liquid Chromatography (HPLC) is characterized by a very rapid separation with extraordinary resolution of peaks. This is achieved by the use of very fine particles and high pressure to maintain an adequate flow rate. Separation can be accomplished in a matter of minutes, or at most an hour. Moreover, only a very small volume of the sample is needed because the particles are so small and close-packed that the void volume is a very small fraction of the bed volume. Also, the concentration of the sample need not be very great because the bands are so narrow that there is very little dilution of the sample.

Affinity Chromatography is a chromatographic procedure that relies on the specific affinity between a substance to be isolated and a molecule that it can specifically bind to. This is a receptor-ligand type interaction. The column material is synthesized by covalently coupling one of the binding partners to an insoluble matrix. The column material is then able to specifically adsorb the substance from the solution. Elution occurs by changing the conditions to those in which binding will not occur (alter pH, ionic strength, temperature, *etc.*).

The matrix should be a substance that itself does not adsorb molecules to any significant extent and that has a broad range of chemical, physical and thermal stability. The ligand should be coupled in such a way as to not affect its binding properties. The ligand should also provide relatively tight binding. And it should be possible to elute the substance without destroying the sample or the ligand. One of the most common forms of affinity chromatography is immunoaffinity chromatography. The generation of antibodies that would be suitable for use in accord with the present invention is discussed below.

C. Immunological Reagents

In the context of the instant invention, it is envisioned that antibodies directed against the claimed peptides may be of relevance. Thus, for certain aspects of the invention, one or more

antibodies may be produced to the expressed antimicrobial peptides. These antibodies may be used in various diagnostic, therapeutic or screening applications.

As used herein, the term "antibody" is intended to refer broadly to any immunologic binding agent such as IgG, IgM, IgA, IgD and IgE. Generally, IgG and/or IgM are preferred because they are the most common antibodies in the physiological situation and because they are most easily made in a laboratory setting.

The term "antibody" is used to refer to any antibody-like molecule that has an antigen binding region, and includes antibody fragments such as Fab', Fab, $F(ab')_2$, single domain antibodies (DABs), Fv, scFv (single chain Fv), and the like. The techniques for preparing and using various antibody-based constructs and fragments are well known in the art. Means for preparing and characterizing antibodies are also well known in the art (See, *e.g.*, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988; incorporated herein by reference).

Monoclonal antibodies (MAbs) are recognized to have certain advantages, *e.g.*, reproducibility and large-scale production, and their use is generally preferred. The invention thus provides monoclonal antibodies of the human, murine, monkey, rat, hamster, rabbit and even chicken origin. Due to the ease of preparation and ready availability of reagents, murine monoclonal antibodies will often be preferred.

However, "humanized" antibodies are also contemplated, as are chimeric antibodies from mouse, rat, or other species, bearing human constant and/or variable region domains, bispecific antibodies, recombinant and engineered antibodies and fragments thereof. Methods for the development of antibodies that are "custom-tailored" to the patient's dental disease are likewise known and such custom-tailored antibodies are also contemplated.

The methods for generating monoclonal antibodies (MAbs) generally begin along the same lines as those for preparing polyclonal antibodies. Briefly, a polyclonal antibody is prepared by immunizing an animal with a LEE or CEE composition in accordance with the present invention and collecting antisera from that immunized animal.

A wide range of animal species can be used for the production of antisera. Typically the animal used for production of antisera is a rabbit, a mouse, a rat, a hamster, a guinea pig or a goat. The choice of animal may be decided upon the ease of manipulation, costs or the desired amount of sera, as would be known to one of skill in the art.

As is also well known in the art, the immunogenicity of a particular immunogen composition can be enhanced by the use of non-specific stimulators of the immune response, known as adjuvants. Suitable adjuvants include all acceptable immunostimulatory compounds,

such as cytokines, chemokines, cofactors, toxins, plasmodia, synthetic compositions or LEEs or CEEs encoding such adjuvants.

Adjuvants that may be used include IL-1, IL-2, IL-4, IL-7, IL-12, γ-interferon, GMCSP, BCG, aluminum hydroxide, MDP compounds, such as thur-MDP and nor-MDP, CGP (MTP-PE), lipid A, and monophosphoryl lipid A (MPL). RIBI, which contains three components extracted from bacteria, MPL, trehalose dimycolate (TDM) and cell wall skeleton (CWS) in a 2% squalene/Tween 80 emulsion is also contemplated. MHC antigens may even be used. Exemplary, often preferred adjuvants include complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis)*, incomplete Freund's adjuvants and aluminum hydroxide adjuvant.

In addition to adjuvants, it may be desirable to coadminister biologic response modifiers (BRM), which have been shown to upregulate T cell immunity or downregulate suppressor cell activity. Such BRMs include, but are not limited to, Cimetidine (CIM; 1200 mg/d) (Smith/Kline, PA); low-dose Cyclophosphamide (CYP; 300 mg/m^2) (Johnson/ Mead, NJ), cytokines such as γ-interferon, IL-2, or IL-12 or genes encoding proteins involved in immune helper functions, such as B7.

The amount of immunogen composition used in the production of polyclonal antibodies varies upon the nature of the immunogen as well as the animal used for immunization. A variety of routes can be used to administer the immunogen including but not limited to subcutaneous, intramuscular, intradermal, intraepidermal, intravenous and intraperitoneal. The production of polyclonal antibodies may be monitored by sampling blood of the immunized animal at various points following immunization.

A second, booster dose (*e.g.*, provided in an injection), may also be given. The process of boosting and titering is repeated until a suitable titer is achieved. When a desired level of immunogenicity is obtained, the immunized animal can be bled and the serum isolated and stored, and/or the animal can be used to generate MAbs.

For production of rabbit polyclonal antibodies, the animal can be bled through an ear vein or alternatively by cardiac puncture. The removed blood is allowed to coagulate and then centrifuged to separate serum components from whole cells and blood clots. The serum may be used as is for various applications or else the desired antibody fraction may be purified by well-known methods, such as affinity chromatography using another antibody, a peptide bound to a solid matrix, or by using, *e.g.*, protein A or protein G chromatography.

MAbs may be readily prepared through use of well-known techniques, such as those exemplified in U.S. Patent 4,196,265, incorporated herein by reference. Typically, this

technique involves immunizing a suitable animal with a selected immunogen composition, *e.g.*, a purified or partially purified protein, polypeptide, peptide or domain, be it a wild-type or mutant composition. The immunizing composition is administered in a manner effective to stimulate antibody producing cells.

The methods for generating monoclonal antibodies (MAbs) generally begin along the same lines as those for preparing polyclonal antibodies. Rodents such as mice and rats are preferred animals, however, the use of rabbit, sheep or frog cells is also possible. The use of rats may provide certain advantages, but mice are preferred, with the BALB/c mouse being most preferred as this is most routinely used and generally gives a higher percentage of stable fusions.

The animals are injected with antigen, generally as described above. The antigen may be mixed with adjuvant, such as Freund's complete or incomplete adjuvant. Booster administrations with the same antigen or DNA encoding the antigen would occur at approximately two-week intervals.

Following immunization, somatic cells with the potential for producing antibodies, specifically B lymphocytes (B cells), are selected for use in the MAb generating protocol. These cells may be obtained from biopsied spleens, tonsils or lymph nodes, or from a peripheral blood sample. Spleen cells and peripheral blood cells are preferred, the former because they are a rich source of antibody-producing cells that are in the dividing plasmablast stage, and the latter because peripheral blood is easily accessible.

Often, a panel of animals will have been immunized and the spleen of an animal with the highest antibody titer will be removed and the spleen lymphocytes obtained by homogenizing the spleen with a syringe. Typically, a spleen from an immunized mouse contains approximately 5×10^7 to 2×10^8 lymphocytes.

The antibody-producing B lymphocytes from the immunized animal are then fused with cells of an immortal myeloma cell, generally one of the same species as the animal that was immunized. Myeloma cell lines suited for use in hybridoma-producing fusion procedures preferably are non-antibody-producing, have high fusion efficiency, and enzyme deficiencies that render then incapable of growing in certain selective media which support the growth of only the desired fused cells (hybridomas).

Any one of a number of myeloma cells may be used, as are known to those of skill in the art (Campbell, 1984). For example, where the immunized animal is a mouse, one may use P3-X63/Ag8, X63-Ag8.653, NS1/1.Ag 4 1, Sp210-Ag14, FO, NSO/U, MPC-11, MPC11-X45-GTG 1.7 and S194/50 Bul; for rats, one may use R210.RCY3, Y3-Ag 1.2.3,

IR983F and 4B210; and U-266, GM1500-GRG2, LICR-LON-HMy2 and UC729-6 are all useful in connection with human cell fusions.

One preferred murine myeloma cell is the NS-1 myeloma cell line (also termed P3-NS-1-Ag4-1), which is readily available from the NIGMS Human Genetic Mutant Cell Repository by requesting cell line repository number GM3573. Another mouse myeloma cell line that may be used is the 8-azaguanine-resistant mouse murine myeloma SP2/0 non-producer cell line.

Methods for generating hybrids of antibody-producing spleen or lymph node cells and myeloma cells usually comprise mixing somatic cells with myeloma cells in a 2:1 proportion, though the proportion may vary from about 20:1 to about 1:1, respectively, in the presence of an agent or agents (chemical or electrical) that promote the fusion of cell membranes. Fusion methods using Sendai virus have been described by Kohler and Milstein (1975; 1976), and those using polyethylene glycol (PEG), such as 37% (v/v) PEG, by Gefter *et al.* (1977). The use of electrically induced fusion methods is also appropriate.

Fusion procedures usually produce viable hybrids at low frequencies, about 1×10^{-6} to 1×10^{-8}. However, this does not pose a problem, as the viable, fused hybrids are differentiated from the parental, unfused cells (particularly the unfused myeloma cells that would normally continue to divide indefinitely) by culturing in a selective medium. The selective medium is generally one that contains an agent that blocks the *de novo* synthesis of nucleotides in the tissue culture media. Exemplary and preferred agents are aminopterin, methotrexate, and azaserine. Aminopterin and methotrexate block *de novo* synthesis of both purines and pyrimidines, whereas azaserine blocks only purine synthesis. Where aminopterin or methotrexate is used, the media is supplemented with hypoxanthine and thymidine as a source of nucleotides (HAT medium). Where azaserine is used, the media is supplemented with hypoxanthine.

The preferred selection medium is HAT. Only cells capable of operating nucleotide salvage pathways are able to survive in HAT medium. The myeloma cells are defective in key enzymes of the salvage pathway, *e.g.*, hypoxanthine phosphoribosyl transferase (HPRT), and they cannot survive. The B cells can operate this pathway, but they have a limited life span in culture and generally die within about two weeks. Therefore, the only cells that can survive in the selective media are those hybrids formed from myeloma and B cells.

This culturing provides a population of hybridomas from which specific hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microtiter plates, followed by testing the individual clonal supernatants (after about two to three weeks) for the desired reactivity. The assay should be sensitive, simple and rapid,

such as radioimmunoassays, enzyme immunoassays, cytotoxicity assays, plaque assays, dot immunobinding assays, and the like.

The selected hybridomas would then be serially diluted and cloned into individual antibody-producing cell lines, which clones can then be propagated indefinitely to provide MAbs. The cell lines may be exploited for MAb production in two basic ways. First, a sample of the hybridoma can be injected (often into the peritoneal cavity) into a histocompatible animal of the type that was used to provide the somatic and myeloma cells for the original fusion (*e.g.*, a syngeneic mouse). Optionally, the animals are primed with a hydrocarbon, especially oils such as pristane (tetramethylpentadecane) prior to injection. The injected animal develops tumors secreting the specific monoclonal antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can then be tapped to provide MAbs in high concentration. Second, the individual cell lines could be cultured *in vitro*, where the MAbs are naturally secreted into the culture medium from which they can be readily obtained in high concentrations.

MAbs produced by either means may be further purified, if desired, using filtration, centrifugation and various chromatographic methods such as HPLC or affinity chromatography. Fragments of the monoclonal antibodies of the invention can be obtained from the monoclonal antibodies so produced by methods which include digestion with enzymes, such as pepsin or papain, and/or by cleavage of disulfide bonds by chemical reduction. Alternatively, monoclonal antibody fragments encompassed by the present invention can be synthesized using an automated peptide synthesizer.

It is also contemplated that a molecular cloning approach may be used to generate monoclonals. In one embodiment, combinatorial immunoglobulin phagemid libraries are prepared from RNA isolated from the spleen of the immunized animal, and phagemids expressing appropriate antibodies are selected by panning using cells expressing the antigen and control cells. The advantages of this approach over conventional hybridoma techniques are that approximately 10^4 times as many antibodies can be produced and screened in a single round, and that new specificities are generated by H and L chain combination which further increases the chance of finding appropriate antibodies. In another example, LEEs or CEEs can be used to produce antigens in vitro with a cell free system. These can be used as targets for scanning single chain antibody libraries. This would enable many different antibodies to be identified very quickly without the use of animals.

Alternatively, monoclonal antibody fragments encompassed by the present invention can be synthesized using an automated peptide synthesizer, or by expression of full-length gene or of gene fragments in *E. coli*.

D. Gene Therapy

In particular embodiments of the instant invention, it is envisioned that antimicrobial peptides and the nucleic acid sequence encoding them may be utilized in gene therapy. For example, individuals immunodeficient due to disease, injury or genetic defect may be administered a nucleic acid construct comprising a genetic sequence encoding the β-defensin antimicrobial peptides.

In certain embodiments of the invention, the nucleic acid encoding the gene may be stably integrated into the genome of the cell. In yet further embodiments, the nucleic acid may be stably maintained in the cell as a separate, episomal segment of DNA. Such nucleic acid segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of or in synchronization with the host cell cycle. How the expression construct is delivered to a cell and where in the cell the nucleic acid remains is dependent on the type of expression construct employed.

1. DNA Delivery Using Viral Vectors

The ability of certain viruses to infect cells or enter cells via receptor-mediated endocytosis, and to integrate into host cell genome and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign genes into mammalian cells. Preferred gene therapy vectors of the present invention will generally be viral vectors.

Although some viruses that can accept foreign genetic material are limited in the number of nucleotides they can accommodate and in the range of cells they infect, these viruses have been demonstrated to successfully effect gene expression. However, adenoviruses do not integrate their genetic material into the host genome and therefore do not require host replication for gene expression, making them ideally suited for rapid, efficient, heterologous gene expression. Techniques for preparing replication-defective infective viruses are well known in the art.

Of course, in using viral delivery systems, one will desire to purify the virion sufficiently to render it essentially free of undesirable contaminants, such as defective interfering viral particles or endotoxins and other pyrogens such that it will not cause any untoward reactions in the cell, animal or individual receiving the vector construct. A preferred means of purifying the

vector involves the use of buoyant density gradients, such as cesium chloride gradient centrifugation.

a. Adenoviral Vectors

A particular method for delivery of the expression constructs involves the use of an adenovirus expression vector. Although adenovirus vectors are known to have a low capacity for integration into genomic DNA, this feature is counterbalanced by the high efficiency of gene transfer afforded by these vectors. "Adenovirus expression vector" is meant to include those constructs containing adenovirus sequences sufficient to (a) support packaging of the construct and (b) to ultimately express a tissue-specific transforming construct that has been cloned therein.

The expression vector comprises a genetically engineered form of adenovirus. Knowledge of the genetic organization or adenovirus, a 36 kb, linear, double-stranded DNA virus, allows substitution of large pieces of adenoviral DNA with foreign sequences up to 7 kb (Grunhaus and Horwitz, 1992). In contrast to retrovirus, the adenoviral infection of host cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal manner without potential genotoxicity. Also, adenoviruses are structurally stable, and no genome rearrangement has been detected after extensive amplification.

Adenovirus is particularly suitable for use as a gene transfer vector because of its mid-sized genome, ease of manipulation, high titer, wide target-cell range and high infectivity. Both ends of the viral genome contain 100-200 base pair inverted repeats (ITRs), which are *cis* elements necessary for viral DNA replication and packaging. The early (E) and late (L) regions of the genome contain different transcription units that are divided by the onset of viral DNA replication. The E1 region (E1A and E1B) encodes proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The expression of the E2 region (E2A and E2B) results in the synthesis of the proteins for viral DNA replication. These proteins are involved in DNA replication, late gene expression and host cell shut-off (Renan, 1990). The products of the late genes, including the majority of the viral capsid proteins, are expressed only after significant processing of a single primary transcript issued by the major late promoter (MLP). The MLP, (located at 16.8 m.u.) is particularly efficient during the late phase of infection, and all the mRNA's issued from this promoter possess a 5'-tripartite leader (TPL) sequence which makes them preferred mRNA's for translation.

In a current system, recombinant adenovirus is generated from homologous recombination between shuttle vector and provirus vector. Due to the possible recombination between two proviral vectors, wild-type adenovirus may be generated from this process.

Therefore, it is critical to isolate a single clone of virus from an individual plaque and examine its genomic structure.

Generation and propagation of the current adenovirus vectors, which are replication deficient, depend on a unique helper cell line, designated 293, which was transformed from human embryonic kidney cells by Ad5 DNA fragments and constitutively expresses E1 proteins (E1A and E1B; Graham *et al.*, 1977). Since the E3 region is dispensable from the adenovirus genome (Jones and Shenk, 1978), the current adenovirus vectors, with the help of 293 cells, carry foreign DNA in either the E1, the D3 or both regions (Graham and Prevec, 1991). In nature, adenovirus can package approximately 105% of the wild-type genome (Ghosh-Choudhury *et al.*, 1987), providing capacity for about 2 extra kb of DNA. Combined with the approximately 5.5 kb of DNA that is replaceable in the E1 and E3 regions, the maximum capacity of the current adenovirus vector is under 7.5 kb, or about 15% of the total length of the vector. More than 80% of the adenovirus viral genome remains in the vector backbone.

Helper cell lines may be derived from human cells such as human embryonic kidney cells, muscle cells, hematopoietic cells or other human embryonic mesenchymal or epithelial cells. Alternatively, the helper cells may be derived from the cells of other mammalian species that are permissive for human adenovirus. Such cells include, *e.g.*, Vero cells or other monkey embryonic mesenchymal or epithelial cells. As stated above, the preferred helper cell line is 293.

Racher *et al.* (1995) discloses improved methods for culturing 293 cells and propagating adenovirus. In one format, natural cell aggregates are grown by inoculating individual cells into 1 liter siliconized spinner flasks (Techne, Cambridge, UK) containing 100-200 ml of medium. Following stirring at 40 rpm, the cell viability is estimated with trypan blue. In another format, Fibra-Cel microcarriers (Bibby Sterlin, Stone, UK) (5 g/l) is employed as follows. A cell inoculum, resuspended in 5 ml of medium, is added to the carrier (50 ml) in a 250 ml Erlenmeyer flask and left stationary, with occasional agitation, for 1 to 4 h. The medium is then replaced with 50 ml of fresh medium and shaking initiated. For virus production, cells are allowed to grow to about 80% confluence, after which time the medium is replaced (to 25% of the final volume) and adenovirus added at an MOI of 0.05. Cultures are left stationary overnight, following which the volume is increased to 100% and shaking commenced for another 72 h.

Other than the requirement that the adenovirus vector be replication defective, or at least conditionally defective, the nature of the adenovirus vector is not believed to be crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known

serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the present invention. This is because Adenovirus type 5 is a human adenovirus about which a great deal of biochemical and genetic information is known, and it has historically been used for most constructions employing adenovirus as a vector.

As stated above, the typical vector according to the present invention is replication defective and will not have an adenovirus E1 region. Thus, it will be most convenient to introduce the transforming construct at the position from which the E1-coding sequences have been removed. However, the position of insertion of the construct within the adenovirus sequences is not critical to the invention. The polynucleotide encoding the gene of interest may also be inserted in lieu of the deleted E3 region in E3 replacement vectors as described by Karlsson *et al.* (1986) or in the E4 region where a helper cell line or helper virus complements the E4 defect.

Adenovirus growth and manipulation is known to those of skill in the art, and exhibits broad host range *in vitro* and *in vivo*. This group of viruses can be obtained in high titers, *e.g.*, 10^9 to 10^{11} plaque-forming units per ml, and they are highly infective. The life cycle of adenovirus does not require integration into the host cell genome. The foreign genes delivered by adenovirus vectors are episomal and, therefore, have low genotoxicity to host cells. No side effects have been reported in studies of vaccination with wild-type adenovirus (Couch *et al.*, 1963; Top *et al.*, 1971), demonstrating their safety and therapeutic potential as *in vivo* gene transfer vectors.

Adenovirus vectors have been used in eukaryotic gene expression (Levrero *et al.*, 1991; Gomez-Foix *et al.*, 1992) and vaccine development (Grunhaus and Horwitz, 1992; Graham and Prevec, 1992). Recently, animal studies suggested that recombinant adenovirus could be used for gene therapy (Stratford-Perricaudet and Perricaudet, 1991; Stratford-Perricaudet *et al.*, 1991; Rich *et al.*, 1993). Studies in administering recombinant adenovirus to different tissues include trachea instillation (Rosenfeld *et al.*, 1991; Rosenfeld *et al.*, 1992), muscle injection (Ragot *et al.*, 1993), peripheral intravenous injections (Herz and Gerard, 1993) and stereotactic inoculation into the brain (Le Gal La Salle *et al.*, 1993). Recombinant adenovirus and adeno-associated virus (see below) can both infect and transduce non-dividing human primary cells.

b. AAV Vectors

Adeno-associated virus (AAV) is an attractive vector system for use in the cell transduction of the present invention as it has a high frequency of integration and it can infect nondividing cells, thus making it useful for delivery of genes into mammalian cells, for example,

in tissue culture (Muzyczka, 1992) or *in vivo*. AAV has a broad host range for infectivity (Tratschin *et al.*, 1984; Laughlin *et al.*, 1986; Lebkowski *et al.*, 1988; McLaughlin *et al.*, 1988). Details concerning the generation and use of rAAV vectors are described in U.S. Patent No. 5,139,941 and U.S. Patent No. 4,797,368, each incorporated herein by reference.

Studies demonstrating the use of AAV in gene delivery include LaFace *et al.* (1988); Zhou *et al.* (1993); Flotte *et al.* (1993); and Walsh *et al.* (1994). Recombinant AAV vectors have been used successfully for *in vitro* and *in vivo* transduction of marker genes (Kaplitt *et al.*, 1994; Lebkowski *et al.*, 1988; Samulski *et al.*, 1989; Yoder *et al.*, 1994; Zhou *et al.*, 1994; Hermonat and Muzyczka, 1984; Tratschin *et al.*, 1984; McLaughlin *et al.*, 1988) and genes involved in human diseases (Flotte *et al.*, 1992; Ohi *et al.*, 1990; Walsh *et al.*, 1994; Wei *et al.*, 1994). Recently, an AAV vector has been approved for phase I human trials for the treatment of cystic fibrosis.

AAV is a dependent parvovirus in that it requires coinfection with another virus (either adenovirus or a member of the herpes virus family) to undergo a productive infection in cultured cells (Muzyczka, 1992). In the absence of coinfection with helper virus, the wild-type AAV genome integrates through its ends into human chromosome 19 where it resides in a latent state as a provirus (Kotin *et al.*, 1990; Samulski *et al.*, 1991). rAAV, however, is not restricted to chromosome 19 for integration unless the AAV Rep protein is also expressed (Shelling and Smith, 1994). When a cell carrying an AAV provirus is superinfected with a helper virus, the AAV genome is "rescued" from the chromosome or from a recombinant plasmid, and a normal productive infection is established (Samulski *et al.*, 1989; McLaughlin *et al.*, 1988; Kotin *et al.*, 1990; Muzyczka, 1992).

Typically, recombinant AAV (rAAV) virus is made by cotransfecting a plasmid containing the gene of interest flanked by the two AAV terminal repeats (McLaughlin *et al.*, 1988; Samulski *et al.*, 1989; each incorporated herein by reference) and an expression plasmid containing the wild-type AAV coding sequences without the terminal repeats, for example pIM45 (McCarty *et al.*, 1991; incorporated herein by reference). The cells are also infected or transfected with adenovirus or plasmids carrying the adenovirus genes required for AAV helper function. rAAV virus stocks made in such fashion are contaminated with adenovirus which must be physically separated from the rAAV particles (for example, by cesium chloride density centrifugation). Alternatively, adenovirus vectors containing the AAV coding regions or cell lines containing the AAV coding regions and some or all of the adenovirus helper genes could be used (Yang *et al.*, 1994; Clark *et al.*, 1995). Cell lines carrying the rAAV DNA as an integrated provirus can also be used (Flotte *et al.*, 1995).

c. Retroviral Vectors

Retroviruses have promise as gene delivery vectors due to their ability to integrate their genes into the host genome, transferring a large amount of foreign genetic material, infecting a broad spectrum of species and cell types and of being packaged in special cell-lines (Miller, 1992).

The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by a process of reverse-transcription (Coffin, 1990). The resulting DNA then stably integrates into cellular chromosomes as a provirus and directs synthesis of viral proteins. The integration results in the retention of the viral gene sequences in the recipient cell and its descendants. The retroviral genome contains three genes, gag, pol, and env that code for capsid proteins, polymerase enzyme, and envelope components, respectively. A sequence found upstream from the gag gene contains a signal for packaging of the genome into virions. Two long terminal repeat (LTR) sequences are present at the 5' and 3' ends of the viral genome. These contain strong promoter and enhancer sequences and are also required for integration in the host cell genome (Coffin, 1990).

In order to construct a retroviral vector, a nucleic acid encoding a gene of interest is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing the gag, pol, and env genes but without the LTR and packaging components is constructed (Mann *et al.*, 1983). When a recombinant plasmid containing a cDNA, together with the retroviral LTR and packaging sequences is introduced into this cell line (by calcium phosphate precipitation for example), the packaging sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media (Nicolas and Rubenstein, 1988; Temin, 1986; Mann *et al.*, 1983). The media containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are able to infect a broad variety of cell types. However, integration and stable expression require the division of host cells (Paskind *et al.*, 1975).

Concern with the use of defective retrovirus vectors is the potential appearance of wild-type replication-competent virus in the packaging cells. This can result from recombination events in which the intact sequence from the recombinant virus inserts upstream from the gag, pol, env sequence integrated in the host cell genome. However, new packaging cell lines are now available that should greatly decrease the likelihood of recombination (Markowitz *et al.*, 1988; Hersdorffer *et al.*, 1990).

Gene delivery using second generation retroviral vectors has been reported. Kasahara *et al.* (1994) prepared an engineered variant of the Moloney murine leukemia virus, that normally infects only mouse cells, and modified an envelope protein so that the virus specifically bound to, and infected, human cells bearing the erythropoietin (EPO) receptor. This was achieved by inserting a portion of the EPO sequence into an envelope protein to create a chimeric protein with a new binding specificity.

d. Other Viral Vectors

Other viral vectors may be employed as expression constructs in the present invention. Vectors derived from viruses such as vaccinia virus (Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988), sindbis virus, cytomegalovirus and herpes simplex virus may be employed. They offer several attractive features for various mammalian cells (Friedmann, 1989; Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988; Horwich *et al.*, 1990).

With the recent recognition of defective hepatitis B viruses, new insight was gained into the structure-function relationship of different viral sequences. *In vitro* studies showed that the virus could retain the ability for helper-dependent packaging and reverse transcription despite the deletion of up to 80% of its genome (Horwich *et al.*, 1990). This suggested that large portions of the genome could be replaced with foreign genetic material. Chang *et al.* recently introduced the chloramphenicol acetyltransferase (CAT) gene into duck hepatitis B virus genome in the place of the polymerase, surface, and pre-surface coding sequences. It was cotransfected with wild-type virus into an avian hepatoma cell line. Culture media containing high titers of the recombinant virus were used to infect primary duckling hepatocytes. Stable CAT gene expression was detected for at least 24 days after transfection (Chang *et al.*, 1991).

In certain further embodiments, the gene therapy vector will be HSV. A factor that makes HSV an attractive vector is the size and organization of the genome. Because HSV is large, incorporation of multiple genes or expression cassettes is less problematic than in other smaller viral systems. In addition, the availability of different viral control sequences with varying performance (temporal, strength, *etc.*) makes it possible to control expression to a greater extent than in other systems. It also is an advantage that the virus has relatively few spliced messages, further easing genetic manipulations. HSV also is relatively easy to manipulate and can be grown to high titers. Thus, delivery is less of a problem, both in terms of volumes needed to attain sufficient MOI and in a lessened need for repeat dosings.

e. Modified Viruses

In still further embodiments of the present invention, the nucleic acids to be delivered are housed within an infective virus that has been engineered to express a specific binding ligand. The virus particle will thus bind specifically to the cognate receptors of the target cell and deliver the contents to the cell. A novel approach designed to allow specific targeting of retrovirus vectors was recently developed based on the chemical modification of a retrovirus by the chemical addition of lactose residues to the viral envelope. This modification can permit the specific infection of hepatocytes via sialoglycoprotein receptors.

Another approach to targeting of recombinant retroviruses was designed in which biotinylated antibodies against a retroviral envelope protein and against a specific cell receptor were used. The antibodies were coupled *via* the biotin components by using streptavidin (Roux *et al.*, 1989). Using antibodies against major histocompatibility complex class I and class II antigens, they demonstrated the infection of a variety of human cells that bore those surface antigens with an ecotropic virus *in vitro* (Roux *et al.*, 1989).

2. Other Methods of DNA Delivery

In various embodiments of the invention, DNA is delivered to a cell as an expression construct. In order to effect expression of a gene construct, the expression construct must be delivered into a cell. As described herein, the preferred mechanism for delivery is *via* viral infection, where the expression construct is encapsidated in an infectious viral particle. However, several non-viral methods for the transfer of expression constructs into cells also are contemplated by the present invention. In one embodiment of the present invention, the expression construct may consist only of naked recombinant DNA or plasmids. Transfer of the construct may be performed by any of the methods mentioned which physically or chemically permeabilize the cell membrane. Some of these techniques may be successfully adapted for *in vivo* or *ex vivo* use, as discussed below.

a. Liposome-Mediated Transfection

In a further embodiment of the invention, the expression construct may be entrapped in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and

Bachhawat, 1991). Also contemplated is an expression construct complexed with Lipofectamine (Gibco BRL).

Liposome-mediated nucleic acid delivery and expression of foreign DNA *in vitro* has been very successful (Nicolau and Sene, 1982; Fraley *et al.*, 1979; Nicolau *et al.*, 1987). Wong *et al.* (1980) demonstrated the feasibility of liposome-mediated delivery and expression of foreign DNA in cultured chick embryo, HeLa and hepatoma cells.

In certain embodiments of the invention, the liposome may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA (Kaneda *et al.*, 1989). In other embodiments, the liposome may be complexed or employed in conjunction with nuclear non-histone chromosomal proteins (HMG-1) (Kato *et al.*, 1991). In yet further embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-1. In other embodiments, the delivery vehicle may comprise a ligand and a liposome. Where a bacterial promoter is employed in the DNA construct, it also will be desirable to include within the liposome an appropriate bacterial polymerase.

b. Electroporation

In certain embodiments of the present invention, the expression construct is introduced into the cell *via* electroporation. Electroporation involves the exposure of a suspension of cells and DNA to a high-voltage electric discharge.

Transfection of eukaryotic cells using electroporation has been quite successful. Mouse pre-B lymphocytes have been transfected with human kappa-immunoglobulin genes (Potter *et al.*, 1984), and rat hepatocytes have been transfected with the chloramphenicol acetyltransferase gene (Tur-Kaspa *et al.*, 1986) in this manner.

c. Calcium Phosphate Precipitation or DEAE-Dextran Treatment

In other embodiments of the present invention, the expression construct is introduced to the cells using calcium phosphate precipitation. Human KB cells have been transfected with adenovirus 5 DNA (Graham and Van Der Eb, 1973) using this technique. Also in this manner, mouse L(A9), mouse C127, CHO, CV-1, BHK, NIH3T3 and HeLa cells were transfected with a neomycin marker gene (Chen and Okayama, 1987), and rat hepatocytes were transfected with a variety of marker genes (Rippe *et al.*, 1990).

In another embodiment, the expression construct is delivered into the cell using DEAE-dextran followed by polyethylene glycol. In this manner, reporter plasmids were introduced into mouse myeloma and erythroleukemia cells (Gopal, 1985).

d. Particle Bombardment

Another embodiment of the invention for transferring a naked DNA expression construct into cells may involve particle bombardment. This method depends on the ability to accelerate DNA-coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein *et al.*, 1987). Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force (Yang *et al.*, 1990). The microprojectiles used have consisted of biologically inert substances such as tungsten or gold beads.

e. Direct Microinjection or Sonication Loading

Further embodiments of the present invention include the introduction of the expression construct by direct microinjection or sonication loading. Direct microinjection has been used to introduce nucleic acid constructs into *Xenopus* oocytes (Harland and Weintraub, 1985), and LTK$^-$ fibroblasts have been transfected with the thymidine kinase gene by sonication loading (Fechheimer *et al.*, 1987).

f. Adenoviral Assisted Transfection

In certain embodiments of the present invention, the expression construct is introduced into the cell using adenovirus assisted transfection. Increased transfection efficiencies have been reported in cell systems using adenovirus coupled systems (Kelleher and Vos, 1994; Cotten *et al.*, 1992; Curiel, 1994).

g. Receptor Mediated Transfection

Still further expression constructs that may be employed to deliver the tissue-specific promoter and transforming construct to the target cells are receptor-mediated delivery vehicles. These take advantage of the selective uptake of macromolecules by receptor-mediated endocytosis that will be occurring in the target cells. In view of the cell type-specific distribution of various receptors, this delivery method adds another degree of specificity to the present invention. Specific delivery in the context of another mammalian cell type is described by Wu and Wu (1993; incorporated herein by reference).

Certain receptor-mediated gene targeting vehicles comprise a cell receptor-specific ligand and a DNA-binding agent. Others comprise a cell receptor-specific ligand to which the DNA construct to be delivered has been operatively attached. Several ligands have been used for receptor-mediated gene transfer (Wu and Wu, 1987; Wagner *et al.*, 1990; Perales *et al.*, 1994;

Myers, EPO 0273085), which establishes the operability of the technique. In the context of the present invention, the ligand will be chosen to correspond to a receptor specifically expressed on the neuroendocrine target cell population.

In other embodiments, the DNA delivery vehicle component of a cell-specific gene targeting vehicle may comprise a specific binding ligand in combination with a liposome. The nucleic acids to be delivered are housed within the liposome and the specific binding ligand is functionally incorporated into the liposome membrane. The liposome will thus specifically bind to the receptors of the target cell and deliver the contents to the cell. Such systems have been shown to be functional using systems in which, for example, epidermal growth factor (EGF) is used in the receptor-mediated delivery of a nucleic acid to cells that exhibit upregulation of the EGF receptor.

In still further embodiments, the DNA delivery vehicle component of the targeted delivery vehicles may be a liposome itself, which will preferably comprise one or more lipids or glycoproteins that direct cell-specific binding. For example, Nicolau *et al.* (1987) employed lactosyl-ceramide, a galactose-terminal asialganglioside, incorporated into liposomes and observed an increase in the uptake of the insulin gene by hepatocytes. It is contemplated that the tissue-specific transforming constructs of the present invention can be specifically delivered into the target cells in a similar manner.

E. Pharmaceutical Compositions

1. Pharmaceutically Acceptable Carriers

Aqueous compositions of the present invention comprise an effective amount of the β-defensin protein, peptide, epitopic core region, inhibitor, nucleic acid sequence or such like, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Aqueous compositions of gene therapy vectors expressing any of the foregoing are also contemplated. The phrases "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, or a human, as appropriate.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions. For human administration,

preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

The biological material should be extensively dialyzed to remove undesired small molecular weight molecules and/or lyophilized for more ready formulation into a desired vehicle, where appropriate. The active compounds will then generally be formulated for parenteral administration, *e.g.*, formulated for injection via the intravenous, intramuscular, subcutaneous, intralesional, or even intraperitoneal routes. The preparation of an aqueous composition that contains a β-defensin agent as an active component or ingredient will be known to those of skill in the art in light of the present disclosure. Typically, such compositions can be prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for using to prepare solutions or suspensions upon the addition of a liquid prior to injection can also be prepared; and the preparations can also be emulsified.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

An β-defensin protein or peptide of the present invention can be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. In terms of using peptide therapeutics as active ingredients, the technology of U.S. Patents 4,608,251; 4,601,903; 4,599,231; 4,599,230; 4,596,792; and 4,578,770, each incorporated herein by reference, may be used.

The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The preparation of more, or highly, concentrated solutions for direct injection is also contemplated, where the use of DMSO as solvent is envisioned to result in extremely rapid penetration, delivering high concentrations of the active agents to a small tumor area.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and the like can also be employed.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the

condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

The active β-defensin peptide or agents may be formulated within a therapeutic mixture to comprise about 0.0001 to 1.0 milligrams, or about 0.001 to 0.1 milligrams, or about 0.1 to 1.0 or even about 10 milligrams per dose or so. Multiple doses can also be administered.

In addition to the compounds formulated for parenteral administration, such as intravenous or intramuscular injection, other pharmaceutically acceptable forms include, *e.g.*, tablets or other solids for oral administration; liposomal formulations; time release capsules; and any other form currently used, including cremes.

One may also use nasal solutions or sprays, aerosols or inhalants in the present invention. Nasal solutions are usually aqueous solutions designed to be administered to the nasal passages in drops or sprays. Nasal solutions are prepared so that they are similar in many respects to nasal secretions, so that normal ciliary action is maintained. Thus, the aqueous nasal solutions usually are isotonic and slightly buffered to maintain a pH of 5.5 to 6.5. In addition, antimicrobial preservatives, similar to those used in ophthalmic preparations, and appropriate drug stabilizers, if required, may be included in the formulation. Various commercial nasal preparations are known and include, for example, antibiotics and antihistamines and are used for asthma prophylaxis.

Additional formulations which are suitable for other modes of administration include vaginal suppositories and pessaries. A rectal pessary or suppository may also be used. Suppositories are solid dosage forms of various weights and shapes, usually medicated, for insertion into the rectum, vagina or the urethra. After insertion, suppositories soften, melt or dissolve in the cavity fluids. In general, for suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1%-2%.

Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders. In certain defined embodiments, oral pharmaceutical compositions will comprise an inert diluent or assimilable edible carrier, or they may be enclosed in hard or soft shell gelatin capsule, or they may be compressed into tablets, or they may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tables, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 0.1% of

active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 2 to about 75% of the weight of the unit, or preferably between 25-60%. The amount of active compounds in such therapeutically useful compositions is such that a suitable dosage will be obtained.

The tablets, troches, pills, capsules and the like may also contain the following: a binder, as gum tragacanth, acacia, cornstarch, or gelatin; excipients, such as dicalcium phosphate; a disintegrating agent, such as corn starch, potato starch, alginic acid and the like; a lubricant, such as magnesium stearate; and a sweetening agent, such as sucrose, lactose or saccharin may be added or a flavoring agent, such as peppermint, oil of wintergreen, or cherry flavoring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup of elixir may contain the active compounds sucrose as a sweetening agent methyl and propylparabens as preservatives, a dye and flavoring, such as cherry or orange flavor.

2. Liposomes and Nanocapsules

In certain embodiments, the use of liposomes and/or nanoparticles is contemplated for the introduction of β-defensin protein, peptides or agents, or gene therapy vectors into host cells. The formation and use of liposomes is generally known to those of skill in the art, and is also described below.

Nanocapsules can generally entrap compounds in a stable and reproducible way. To avoid side effects due to intracellular polymeric overloading, such ultrafine particles (sized around 0.1 μm) should be designed using polymers able to be degraded *in vivo*. Biodegradable polyalkyl-cyanoacrylate nanoparticles that meet these requirements are contemplated for use in the present invention, and such particles may be are easily made.

Liposomes are formed from phospholipids that are dispersed in an aqueous medium and spontaneously form multilamellar concentric bilayer vesicles (also termed multilamellar vesicles (MLVs). MLVs generally have diameters of from 25 nm to 4 μm. Sonication of MLVs results in the formation of small unilamellar vesicles (SUVs) with diameters in the range of 200 to 500 Å, containing an aqueous solution in the core.

The following information may also be utilized in generating liposomal formulations. Phospholipids can form a variety of structures other than liposomes when dispersed in water, depending on the molar ratio of lipid to water. At low ratios the liposome is the preferred structure. The physical characteristics of liposomes depend on pH, ionic strength and the

presence of divalent cations. Liposomes can show low permeability to ionic and polar substances, but at elevated temperatures undergo a phase transition which markedly alters their permeability. The phase transition involves a change from a closely packed, ordered structure, known as the gel state, to a loosely packed, less-ordered structure, known as the fluid state. This occurs at a characteristic phase-transition temperature and results in an increase in permeability to ions, sugars and drugs.

Liposomes interact with cells via four different mechanisms: Endocytosis by phagocytic cells of the reticuloendothelial system such as macrophages and neutrophils; adsorption to the cell surface, either by nonspecific weak hydrophobic or electrostatic forces, or by specific interactions with cell-surface components; fusion with the plasma cell membrane by insertion of the lipid bilayer of the liposome into the plasma membrane, with simultaneous release of liposomal contents into the cytoplasm; and by transfer of liposomal lipids to cellular or subcellular membranes, or *vice versa*, without any association of the liposome contents. Varying the liposome formulation can alter which mechanism is operative, although more than one may operate at the same time.

F. Therapeutic and Antiseptic Uses

The instant invention comprises a composition and methods for its use in the prevention of microbial growth. It is envisioned that the peptides may be delivered in a composition by themselves or in combination with any one or more additional antimicrobial agents to produce a complementary or synergistic effect. In a further embodiment, the invention also encompasses methods to reduce antimicrobial resistance, caused by any of the seven mechanisms described by Davies (1986) (previously cited), using an antimicrobial peptide and one or more antimicrobial agents or antibiotics. Exemplary bacterial strains that have developed antibiotic resistance by one or more of these mechanisms are set forth in Table 4 (Lorian, 1991).

The antimicrobial peptides have broad spectrum antimicrobial properties effective against both Gram-positive and Gram-negative strains of bacteria and are thus frequently effective to kill strains previously deemed multiply drug resistant. The purified antimicrobial peptides may be used without further modifications or may be diluted in a pharmaceutically acceptable carrier. Because of the stability of the peptides it is contemplated that the invention may be administered to humans or animals, included in food preparations, pharmaceutical preparations, medicinal and pharmaceutical products, cosmetic products, hygienic products, cleaning products and cleaning agents, as well as any material to which the peptides could be sprayed on or adhered to wherein the inhibition of microbial growth on such a material is desired.

In the context of medical devices, it is envisioned that the peptides in their pure form or combined with other antimicrobial peptides or agents, could be sprayed on, coated on, or adhered to any surface of a medical device wherein the inhibition of microbial growth on such a surface is desired. Examples of such medical devices include but are not limited to endotracheal tube, a vascular catheter, an urinary catheter, a nephrostomy tube, a biliary stent, a peritoneal catheter, an epidural catheter, a central nervous system catheter, an orthopedic device, a prosthetic valve, and a medical implant. The vascular catheter may be a central venous catheter, an arterial line, an pulmonary artery catheter, and a peripheral venous catheter. The central nervous system catheter may be an intraventricular shunt. Other medical devices that can benefit from the present invention include blood exchanging devices, vascular access ports, cardiovascular catheters, extracorpeal circuits, stents, implantable prostheses, vascular grafts, pumps, heart valves, and cardiovascular sutures, to name a few. Regardless of detailed embodiments, applicability of the invention should not be considered limited with respect to the type of medical device, implant location or materials of construction of the device.

In the context of routes of administration, delivery or application, it is envisioned that the antimicrobial peptides will be delivered in a composition that facilitates the maintenance of the antimicrobial properties of the peptides. For exmple, if the antimicrobial peptides are to be topically administered or placed in hygienic products, cleaning products and cleaning agents, they will be administered in diluent that is properly formulated to retain the proper conformation of the peptides. Due to their immuno-modulatory chemoattractant properties, β-defensins might be used to augment host defense at mucosal surfaces (Yang, *et al.* 1999).

The proper dosage of an antimicrobial peptide necessary to prevent microbial growth and proliferation depends upon a number of factors including the types of bacteria that might be present, the environment into which the peptide is being introduced, and the time that the peptide is envisioned to remain in a given area.

It is further contemplated that the antimicrobial peptides of the invention may be used in combination with or to enhance the activity of other antimicrobial agents or antibiotics. Combinations of the peptides with other agents may be useful to allow antibiotics to be used at lower doses due to toxicity concerns, to enhance the activity of antibiotics whose efficacy has been reduced or to effectuate a synergism between the components such that the combination is more effective than the sum of the efficacy of either component independently. Antibiotics which may be combined with an antimicrobial peptide in combination therapy include but are not limited to penicillin, ampicillin, amoxycillin, vancomycin, cycloserine, bacitracin, cephalolsporin, methicillin, streptomycin, kanamycin, tobramycin, gentamicin, tetracycline,

chlortetracycline, doxycycline, chloramphenicol, lincomycin, clindamycin, erythromycin, oleandomycin, polymyxin nalidixic acid, rifamycin, rifampicin, gantrisin, trimethoprim, isoniazid, paraaminosalicylic acid, and ethambutol. Table 5 (Reese and Betts, 1993), lists the antibiotics generally preferred for use against a given pathogenic bacterium. It is contemplated that the effectiveness of all the antibiotics listed in Table 5 will be increased upon combination with an antimicrobial peptide. Table 6 (Reese and Betts, 1993), itemizes the common pathogenic bacteria that are implicated in focal infections. The present invention is thus contemplated for use against all such infections.

TABLE 4

MECHANISMS OF RESISTANCE TO ANTIMICROBIAL AGENTS

Antimicrobial Agent	**Mechanisms Causing Resistance**	**EXAMPLES OF ORGANISMS**
Aminoglycosides	Modifying enzymes: acetyltransferases, adenylyl-transferases (nucleotidyl-transferases), phosphotransferases	Enterobacteriaceae, *P. aeruginosa, S. aureus, E. faecalis*
	Ribosomal resistance (streptomycin, spectinomycin)	*E.faecalis,* Enterobacteriaceae, *M. tuberculosis, P. aeruginosa*
	Inadequate drug transport	*E. faecalis, P. aeruginosa,* anaerobes
β-Lactams	Enzymatic inactivation	*S. aureus, E. faecalis,* Enterobacteriaceae, *P. aeruginosa, Neisseria* spp., *H. influenzae*
	Low affinity PBPs	*S. pneumoniae, N. gonorrhoeae, S. aureus,*
	Lack of penetration through outer membrane	*P. aeruginosa* *P. aeruginosa,* Enterobacteriaceae
Chloramphenicol	Acetylation	Enterobacteriaceae, *S. aureus,* streptococci, *Bacteroides uniformis*
	Lack of penetration	*P. aeruginosa*
Clindamycin, erythromycin, lincomycin	Ribosomal resistance due to methylation of rRNA	Streptococci, *E. faecalis,*
	Inactivation by esterase	Enterobacteriaceae
	Decreased penetration	Enterobacteriaceae

Antimicrobial Agent	Mechanisms Causing Resistance	EXAMPLES OF ORGANISMS
		S. hominis
Fluoroquinolones	Decreased uptake Altered target site (DNA gyrase)	Enterobacteriaceae, *P. aeruginosa*, staphylococci Enterobacteriaceae, *P. aeruginosa*
Lincomycin	Inactivation	*S. aureus*

Antimicrobial Agent	Mechanisms Causing Resistance	EXAMPLES OF ORGANISMS
Sulfonamides	Synthesis of an altered or alternative target site (dihydropteroate synthetase) Lack of penetration Overproduction of PABA	Enterobacteriaceae, *Neisseria* spp., *P. aeruginosa* Anaerobes *Neisseria, S. aureus*
Tetracycline	Drug efflux Protection of ribosome from tetracycline Inactivation	Enterobacteriaceae, staphylococci, streptococci Streptococci, *E. faecalis*, *Neisseria* spp., *Mycoplasma* spp. Cryptic gene found in *B. fragilis*, expressed resistance in *E. coli*
Trimethoprim	Synthesis of an altered or alternative target site (dihydrofolate reductase) Lack of penetration Ability to use alternative pathway Overproduction of dihydrofolate reductase	Enterobacteriaceae, *V. cholerae*, staphylococci *P. aeruginosa* Enterococci *H. influenzae*
Vancomycin	? ?Blocking of target site	Pediococci, *Leuconostoc* spp. (intrinsic) Enterococci (acquired)

TABLE 5

ANTIBIOTICS OF CHOICE FOR COMMON PATHOGENS

Pathogen	Antibiotic of First Choice[a]	Alternative Agents[a]
Gram-positive cocci		
Staphylococcus aureus or *S. epidermidis*		
Non-penicillinase-producing	Penicillin	A first-generation cephalosporin, vancomycin, imipenem, or clindamycin; a fluoroquinolone[b]
Penicillinase-producing	Penicillinase-resistant penicillin (*e.g.*, oxacillin or nafcillin)	A first-generation cephalosporin, vancomycin, clindamycin, imipenem, amoxicillin-clavulanic acid, ticarcillin-clavulanic acid, ampicillin-sulbactam; a fluoroquinolone[b]
Methicillin-resistant	Vancomycin with or without gentamicin and/or rifampin	TMP-SMZ, minocycline
Streptococci		
Group A, C, G	Penicillin	A cephalosporin[a], vancomycin, erythromycin; clarithromycin; azithromycin; clindamycin
Group B	Penicillin (or ampicillin)	A cephalosporin[a], vancomycin, or erythromycin
Enterococcus		
Endocarditis or other serious infection	Penicillin (or ampicillin) with gentamicin	Vancomycin with gentamicin
Uncomplicated urinary tract infection	Ampicillin or amoxicillin	A fluoroquinolone, nitrofurantoin
Viridans group	Penicillin G (with or without gentamicin)	A cephalosporin[a], vancomycin
S. bovis	Penicillin G	A cephalosporin[a], vancomycin
S. pneumoniae	Penicillin G	A cephalosporin[a], erythromycin, chloramphenicol, vancomycin

Gram-negative cocci		
Neisseria gonorrhoeae	Ceftriaxone	Spectinomycin, a fluoroquinolone, cefoxitin, cefixime, cefotaxime (see Appendix E)
N. meningitidis	Penicillin G	Third-generation cephalosporin, chloramphenicol
Moraxella (Branhamella) catarrhalis	TMP-SMZ	Amoxicillin-clavulanic acid; an erythromycin; clarithromycin azithromycin, cefuroxime, cefixime, third-generation cephalosporin, tetracycline
Gram-positive bacilli		
Clostridium perfringens (and *Clostridium* sp.)	Penicillin G	Chloramphenicol, metronidazole, or clindamycin
Listeria monocytogenes	Ampicillin with or without gentamicin	TMP-SMZ
Gram-negative bacilli		
Acinetobacter	Imipenem	Tobramycin, gentamicin, or amikacin, usually with ticarcillin or piperacillin (or similar agent); TMP-SMZ
Aeromonas hydrophila	TMP-SMZ	Gentamicin, tobramycin; imipenem; a fluoroquinolone
Bacteroides *Bacteroides* sp. (oropharyngeal)	Penicillin G	Clindamycin, cefoxitin, metronidazole, chloramphenicol, cefotetan, ampicillin-sulbactam
B. fragilis strains (gastrointestinal strains)	Metronidazole	Clindamycin; ampicillin-sulbactam; imipenem; cefoxitin[c]; cefotetan[c]; ticarcillin-clavulanic acid; piperacillin[c]; chloramphenicol; cefmetazole[c]
Campylobacter fetus, jejuni	A fluoroquinolone (adults) or an erythromycin	A tetracycline, gentamicin

Enterobacter sp.	Imipenem	An aminoglycoside and piperacillin or ticarcillin or mezlocillin; a third-generation cephalosporin[d]; TMP-SMZ; aztreonam; a fluoroquinolone
Escherichia coli Uncomplicated urinary tract infection Recurrent or systemic infection	TMP-SMZ A cephalosporin[e]	A cephalosporin or a fluoroquinolone Ampicillin with or without an aminoglycoside, TMP-SMZ, oral fluoroquinolones useful in recurrent infections, ampicillin-sulbactam, ticarcillin-clavulanic acid, aztreonam
Haemophilus influenzae (coccobacillary) Life-threatening infections Upper respiratory infections and bronchitis	Cefotaxime or ceftriaxone TMP-SMZ	Chloramphenicol; cefuroxime for pneumonia) Ampicillin or amoxicillin; cefuroxime; a sulfonamide with or without an erythromycin; cefuroxime-axetil; third-generation cephalosporin, amoxicillin-clavulanic acid, cefaclor, tetracycline; clarithromycin; azithromycin
Klebsiella pneumoniae	A cephalosporin[e]	An aminoglycoside, imipenem, TMP-SMZ, ticarcillin-clavulanic acid, ampicillin-sulbactam, aztreonam, a fluoroquinolone; amoxicillin-clavulanic acid
Legionella spp.	Erythromycin with rifampin	TMP-SMZ; clarithromycin; azithromycin; ciprofloxacin
Pasteurella multocida	Penicillin G	Tetracycline, cefuroxime, amoxicillin-clavulanic acid, ampicillin-sulbactam

Proteus sp.	Cefotaxime, ceftizoxime, or ceftriaxone[f]	An aminoglycoside; ticarcillin or piperacillin or mezlocillin; TMP-SMZ; amoxicillin-clavulanic acid; ticarcillin-clavulanic acid, ampicillin-sulbactam; a fluoroquinolone; aztreonam; imipenem
Providencia stuartii	Cefotaxime, ceftizoxime, or ceftriaxone[f]	Imipenem; an aminoglycoside often combined with ticarcillin or piperacillin or similar agent; ticarcillin-clavulanic acid; TMP-SMZ, a fluoroquinolone; aztreonam
Pseudomonas aeruginosa (nonurinary tract infection)	Gentamicin or tobramycin or amikacin (combined with ticarcillin, piperacillin, *etc.* for serious infections) Ciprofloxacin	An aminoglycoside and ceftazidime; imipenem, or aztreonam plus an aminoglycoside; ciprofloxacin
(urinary tract infections)		Carbenicillin; ticarcillin, piperacillin, or mezlocillin; ceftazidime; imipenem; aztreonam; an aminoglycoside
Pseudomonas cepacia	TMP-SMZ	Ceftazidime, chloramphenicol
Salmonella typhi	Ceftriaxone	Ampicillin, amoxicillin, TMP-SMZ, chloramphenicol; a fluoroquinolone
Other species	Cefotaxime or ceftriaxone	Ampicillin or amoxicillin, TMP-SMZ, chloramphenicol; a fluoroquinolone
Serratia	Cefotaxime, ceftizoxime, or ceftriaxone[f]	Gentamicin or amikacin; imipenem; TMP-SMZ; ticarcillin, piperacillin, or mezlocillin; aztreonam; a fluoroquinolone
Shigella	A fluoroquinolone	TMP-SMZ; ceftriaxone; ampicillin

Vibrio cholerae (chlorea)	A tetracycline	TMP-SMZ; a fluoroquinolone
Vibrio vulnificus	A tetracycline	Cefotaxime
Xanthomonas (Pseudomonas) maltophilia	TMP-SMZ	Minocycline, ceftazidime, a fluoroquinolone
Yersinia enterocolitica	TMP-SMZ	A fluoroquinolone; an aminoglycoside; cefotaxime or ceftizoxime
Yersinia pestis (plague)	Streptomycin	A tetracycline; chloramphenicol; gentamicin

Key: TMP-SMZ = trimethoprim-sulfamethoxazole.

[a] Choice presumes susceptibility studies indicate that the pathogen is susceptible to the agent.

[b]The experience with fluoroquinolone use in staphylococcal infections is relatively limited. The fluoroquinolones should be used only in adults.

[c]Up to 15-20% of strains may be resistant.

[d]*Enterobacter spp.* may develop resistance to the cephalosporins.

[e]Specific choice will depend on susceptibility studies. Third-generation cephalosporins may be exquisitely active against many Gram-negative bacilli (*e.g.*, *E. coli*, *Klebsiella* sp.). In some geographic areas, 20-25% of community-acquired *E. coli* infections may be resistant to ampicillin (amoxicillin).

[f]In severely ill patients, this is often combined with an aminoglycoside while awaiting susceptibility data.

TABLE 6

COMMON PATHOGENS IN FOCAL INFECTIONS

Presumed location of Infection	**Common pathogens**	**Gram stain Characteristics of exudate-if available**
Urinary tract infections	Community-acquired: *Escherichia coli* Recurrent or nosocomial: *E. coli: Klebsiella, Proteus, Pseudomonas* sp. Enterococci	GNB GNB GPC
Intravenous catheter phlebitis and/or sepsis		
Peripheral catheter	*Staphylococcus aureus* or *S. epidermidis* *Klebsiella, Enterobacter, Pseudomonas* sp.	GPC GNB
Hyperalimentation line	*Candida* sp., *S. aureus, S. epidermidis*, enterococci *Klebsiella, Enterobacter* sp., *etc.*	Budding yeast; GPC GNB
Arteriovenous shunt	*S. aureus, S. epidermidis*	GPC
Septic bursitis	*S. aureus*	GPC
Biliary tract	*E. coli, Klebsiella* sp., and enterococci; *Bacteroides fragilis* (in elderly patients), *Clostridia* sp.	
Intra-abdominal abscess, peritonitis, or large bowel perforation; diverticulitis[a]	*E. coli* *B. fragilis* *Klebsiella* sp. (Enterococci)	GNB GNB (thin, irregularly stained) GNB GPC
Burn wounds	Early: *S. aureus*, streptococci Later: Gram-negative bacilli, fungi	
Cellulitis, wound and soft tissue infections	*S. aureus* Streptococci *Clostridium* sp.	GPC GPC GPB
Meningitis	See Appendix C	
Pneumonia	See Appendix D	
Pelvic abscess,	Anaerobic streptococci	GPC

Presumed location of Infection	Common pathogens	Gram stain Characteristics of exudate-if available
postabortal or postpartal	*B. fragilis*	GNB (thin, irregularly stained)
	Clostridium sp.	GPB
	E. coli	GNB
	Enterococci	GPC
Septic arthritis	*S. aureus*	GPC
	Haemophilus influenzae (in children younger than 6 yr)	GNC
	Group B streptococci (in neonates)	GPC
	Gram-negative organisms[b]	GNB
Acute osteomyelitis	*S. aureus*	GPC
	H. influenzae (in children younger than 6 yr)	GNC
	Group B streptococci (in neonates)	GPC
	Gram-negative organisms[b]	GNB

Key: GNB = Gram-negative bacilli; GPC = Gram-positive cocci; GPB = Gram-positive bacilli; GNC = Gram-negative coccobacilli.

[a]The precise role of enterococci in intra-abdominal infections is unclear. In mild to moderate infections, it may not be necessary to provide antibiotic activity against enterococci.

[b]In high-risk patients (*e.g.*, immunocompromised, elderly, IV drug abusers, diabetics, debilitated patients).

To reduce the resistance of a microorganism to an antimicrobial agent, as exemplified by reducing the resistance of a bacterium to an antibiotic, or to kill a microorganism or bacterium, one would generally contact the microorganism or bacterium with an effective amount of the antibiotic or antimicrobial agent in combination with an amount of an antimicrobial peptide effective to inhibit growth of the microorganism or bacterium. In terms of killing or reducing the resistance of a bacterium, one would contact the bacterium with an effective amount of an antibiotic in combination with an amount of an antimicrobial peptide effective to inhibit growth and/or proliferation in the bacterium.

The terms "microbe,""microorganism" and "bacterium" are used for simplicity and it will be understood that the invention is suitable for use against a population of microorganisms, *i.e.*, "bacteria".

In the context of bacterial or microbial infections, a person of ordinary skill would recognize the wide variety of potential pathogens. As an exemplary list, bacterial infections, are deemed to include, but not be limited to, the 83 or more distinct serotypes of pneumococci, streptococci such as *S. pyrogenes*, *S. agalactiae*, *S. equi*, *S. canis*, *S. bovis*, *S. equinus*, *S. anginosus*, *S. sanguis*, *S. salivarius*, *S. mitis*, *S. mutans*, other viridans streptococci, peptostreptococci, other related species of streptococci, enterococci such as *Enterococcus faecalis*, *Enterococcus faecium*, Staphylococci, such as *Staphylococcus epidermidis*, *Staphylococcus aureus*, particularly in the nasopharynx, *Hemophilus influenzae*, pseudomonas species such as *Pseudomonas aeruginosa*, *Pseudomonas pseudomallei*, *Pseudomonas mallei*, brucellas such as *Brucella melitensis*, *Brucella suis*, *Brucella abortus*, *Bordetella pertussis*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Moraxella catarrhalis*, *Corynebacterium diphtheriae*, *Corynebacterium ulcerans*, *Corynebacterium pseudotuberculosis*, *Corynebacterium pseudodiphtheriticum*, *Corynebacterium urealyticum*, *Corynebacterium hemolyticum*, *Corynebacterium equi*, etc. *Listeria monocytogenes*, *Nocardia asteroides*, Bacteroides species, Actinomycetes species, *Treponema pallidum*, Leptospirosa species and related organisms. The invention may also be useful against gram negative bacteria such as *Klebsiella pneumoniae*, *Escherichia coli*, *Proteus*, *Serratia* species, *Acinetobacter*, *Yersinia pestis*, *Francisella tularensis*, *Enterobacter* species, *Bacteriodes* and *Legionella* species and the like. In addition, the invention may prove useful in controlling protozoan or macroscopic infections by organisms such as *Cryptosporidium*, *Isospora belli*, *Toxoplasma gondii*, *Trichomonas vaginalis*, *Cyclospora* species, for example, and for *Chlamydia trachomatis* and other *Chlamydia* infections such as *Chlamydia psittaci*, or *Chlamydia pneumoniae*, for example.

The microorganism, *e.g.*, bacterium, or population thereof, may be contacted either *in vitro* or *in vivo*. Contacting *in vivo* may be achieved by administering to an animal (including a human patient) that has, or is suspected to have a microbial or bacterial infection, a therapeutically effective amount of pharmacologically acceptable antimicrobial peptide formulation in alone or in combination with a therapeutic amount of a pharmacologically acceptable formulation of a antibiotic agent. The invention may thus be employed to treat both systemic and localized microbial and bacterial infections by introducing the combination of agents into the general circulation or by applying the combination, *e.g.*, topically to a specific site, such as a wound or burn, or to the eye, ear or other site of infection.

Where an antimicrobial peptide is used in combination with other antimicrobial agents or antibiotics, an "effective amount of an antimicrobial agent or antibiotic" means an amount, or dose, within the range normally given or prescribed. Such ranges are well established in routine

clinical practice and will thus be known to those of skill in the art. Appropriate oral and parenteral doses and treatment regimens are further detailed herein in Table 7 and Table 8. As this invention provides for enhanced microbial and/or bacterial killing, it will be appreciated that effective amounts of an antimicrobial agent or antibiotic may be used that are lower than the standard doses previously recommended when the antimicrobial or antibiotic is combined with a antimicrobial peptide.

Naturally, in confirming the optimal therapeutic dose for antimicrobial peptides, first animal studies and then clinical trials would be conducted, as is routinely practiced in the art. Animal studies are common in the art and are further described herein (Example 2) and in publications such as Lorian (1991, pp. 746-786, incorporated herein by reference) and Cleeland and Squires (incorporated herein by reference, from within the Lorian text).

The ID_{50}/IC_{50} ratio required for safe use of the proposed inhibitor-antimicrobial peptide or combinations of peptide with other antimicrobial agents will be assessed by determining the ID_{50} (median lethal toxic dosage) and the IC_{50} (median effective therapeutic dosage) in experimental animals. The optimal dose for human subjects is then defined by fine-tuning the range in clinical trials. In the case of ID_{50}, the inhibitor is usually administered to mice or rats (orally or intraperitoneal) at several doses (usually 4-5) in the lethal rage. The dose in mg/kg is plotted against % mortality and the dose at 50% represents the ID_{50} (Klaassen, 1990). The IC_{50} is determined in a similar fashion as described by Cleeland and Squires (1991).

In a clinical trial, the therapeutic dose would be determined by maximizing the benefit to the patient, whilst minimizing any side-effects or associated toxicities. Throughout the detailed examples, various therapeutic ranges are listed. Unless otherwise stated, these ranges refer to the amount of an agent to be administered orally.

In optimizing a therapeutic dose within the ranges disclosed herein, one would not use the upper limit of the range as the starting point in a clinical trial due to patient heterogeneity. Starting with a lower or mid-range dose level, and then increasing the dose will limit the possibility of eliciting a toxic or untoward reaction in any given patient or subset of patients. The presence of some side-effects or certain toxic reactions *per se* would not, of course, limit the utility of the invention, as it is well known that most beneficial drugs also produce a limited amount of undesirable effects in certain patients. Also, a variety of means are available to the skilled practitioner to counteract certain side-effects, such as using vitamin B_{12} in association with N_2O treatment (Ostreicher, 1994).

Zak and Sande (1981) reported on the correlation between the *in vitro* and *in vivo* activity of a 1000 compounds that were randomly screened for antimicrobial activity. The important

finding in this study is that negative *in vitro* data is particularly accurate, with the negative *in vitro* results showing more than a 99% correlation with negative *in vivo* activity. This is meaningful in the context of the present invention as one or more *in vitro* assays will be conducted prior to using any given combination in a clinical setting. Any negative result obtained in such an assay will thus be of value, allowing efforts to be more usefully directed.

In the treatment of animals or human patients with combination therapy, there are various appropriate formulations and treatment regimens that may be used. For example, the antimicrobial peptide and second agent(s) may be administered to an animal simultaneously, *e.g.*, in the form of a single composition that includes the antimicrobial peptide and second agent, or by using at least two distinct compositions. The antimicrobial agent could also be administered to the animal prior to the second agent or the second agent may be given prior to the antimicrobial peptide.

Multiple combinations may also be used, such as more than one antimicrobial peptide used with one second agent or more than one second agent. Different classes second agents and antimicrobial peptides may be combined, naturally following the general guidelines known in the art regarding drug interactions. Typically, between one and about five distinct antimicrobial agents are contemplated for use along with between one and about six antimicrobial peptides.

Further embodiments of the invention include therapeutic kits that comprise, in suitable container means, a pharmaceutical formulation of at least one antimicrobial peptide and a pharmaceutical formulation of at least one antimicrobial agent or antibiotic. The antimicrobial peptide and antimicrobial agent or antibiotic may be contained within a single container means, or a plurality of distinct containers may be employed.

Depending on the circumstances, antimicrobial agents may be employed in oral or parenteral treatment regimens. Appropriate doses are well known to those of skill in the art and are described in various publications, such as (Reese and Betts, 1993; incorporated herein by reference). Table 7 and Table 8 (taken from Reese and Betts, 1993) are included herein to provide ready reference to the currently recommended doses of a variety of antimicrobial agents.

Following are definitions of terms that are used in Table 7 and Table 8: qid (4 times daily), tid (3 times daily), bid (twice daily), qd (once daily), q4h (every 4 hours around the clock), q6h (every 6 hours around the clock) and q8h (every 8 hours around the clock).

TABLE 7:

COMMON ANTIBIOTICS AND USUAL ORAL DOSES

ANTIBIOTIC	DOSAGE
Penicillin V Rugby (generic) V-cillin K	250 mg qid
Dicloxacillin Glenlawn (generic) Dynapen	250 mg qid
Cloxacillin (Tegopen)	250 mg qid
Amoxicillin Rugby (generic) Polymox	250 mg tid
Ampicillin Moore (generic) Polycillin	250 mg qid
Augmentin 250-mg tablets chewables (250 mg) 125-mg (suspension) chewables (125 mg)	tid
Carbenicillin (Geocillin)	382 mg qid (1 tb) 2 tab qid
Cephalexin Rugby (generic) Keflex Rugby (generic) Keflex	250 mg qid 500 mg qid
Cefadroxil Rugby (generic) Duricef	1 gm bid
Cephradine Rugby (generic Velosef Rugby (generic) Velosef	250 mg qid 500 mg qid
Cefaclor Ceclor	250 mg tid
Cefuroxime axetil Ceftin	 125 mg bid 250 mg bid 500 mg bid
Cefixime Suprax	400 mg q24h
Cefprozil	

ANTIBIOTIC	DOSAGE
Cefzil	250 mg q12h
Loracarbef (Lorabid)	200 mg bid
Cefpodoxime proxetil (Vantin)	200 mg bid
Clindamycin Cleocin	300 mg q8h
TMP/SMZ Bactrim Septra (generic)	1 double-strength bid
Trimethoprim Rugby (generic) Proloprim	100 mg bid
Erythromycin (base) Abbott E-mycin (delayed release)	250 mg qid
Erythromycin stearate Rugby (generic)	250 mg qid
Azithromycin Zithromax	1 g once only 500 mg, day 1, plus 250 mg, day 2-5
Clarithromycin Biaxin	250 mg bid 500 mg bid
Tetracycline hydrochloride Mylan Sumycin 250	250 mg qid
Doxycycline Lederle (generic) Vibramycin	100 mg qd (with 200-mg initial load)
Vancomycin Vancocin HCl (oral soln/powder)	Capsules 125 mg q6h PO
Metronidazole Rugby (generic) Flagyl	250 mg qid
Norfloxacin Noroxin	400 mg bid
Ciprofloxacin Cipro	250 mg bid 500 mg bid 750 mg bid
Ofloxacin Floxin	200 mg bid 300 mg bid 400 mg bid
Lomefloxacin Maxaquin	400 mg once qd

TABLE 8

COMMON ANTIBIOTICS AND USUAL PARENTERAL DOSES

ANTIBIOTIC	DOSAGE
Penicillin G	2,400,000 units
Pfizerpen G (Pfizer)	12 million units
Oxacillin	12 g
Prostaphlin (Bristol)	
Nafcillin	12 g
Nafcil (Bristol)	
Ampicillin	6 g
Omnipen (Wyeth)	
Ticarcillin	18 g
Ticar (Beecham)	
Piperacillin	18 g
Pipracil (Lederle)	16 g
Mezlocillin	18 g
Mezlin (Miles)	16 g
Ticarcillin-clavulanate	18 g/0.6 g
Timentin (Beecham)	12 g/0.4 g
Ampicillin-sulbactam	6 g
Unasyn (Roerig)	12 g
Cephalothin	9 g (1.5 g q4h)
Keflin (Lilly)	
Cefazolin	4 g (1 g q6h)
Ancef (SKF)	3 g (1 g q8h)
Cefuroxime	6 g2.25 g (750 mg q8h)
Zinacef (Glaxo)	4.5 g (1.5 g q8h)
Cefamandole	9 g (1.5 g q4h)
Mandol (Lilly)	

ANTIBIOTIC	DOSAGE
Cefoxitin Mefoxin (MSD)	8 g (2 g q6h) 6 g (2 g q8h)
Cefonicid Monicid (SKF)	1 g q12h
Cefotetan Cefotan (Stuart)	2 g q12h
Cefmetazole Zefazone (Upjohn)	2 g q8h
Ceftriaxone Rocephin (Roche)	2 g (2.0 g q24h) 1 g (1.0 g q24h)
Ceftazidime Fortax (Glaxo) Taxicef (SKF) Tozidime (Lilly)	6 g (2 g q8h)
Cefotaxime Claforan (Hoechst)	2 g q6h 2 g q8h
Cefoperazone Cefobid (Pfizer)	8 g (2 g q6h) 6 g (2 g q8h)
Ceftizoxime Ceftizox (SKF)	(2 g q8h)
Aztreonam Azactam (Squibb)	2 g q8h 1 g q8h
Imipenem Primaxin (MSD)	2000 mg (500 mg 16h)
Gentamicin Garamycin (Schering) (generic) (Elkins-Sinn)	360 mg (1.5 mg/kg q8h for an 80-kg patient)
Tobramycin Nebcin (Dista)	360 mg (1.5 mg/kg q8h for an 80-kg patient)
Amikacin Amikin (Bristol)	1200 mg (7.5 mg/kg q12h for an 80-kg

ANTIBIOTIC	DOSAGE
	patient)
Clindamycin Cleocin (Upjohn)	2400 mg (600 mg q6h) 2700 mg (900 mg q8h) 1800 mg (600 mg q8h)
Chloramphenicol Chloromycetin (P/D)	4 g (1 g q6h)
TMP/SMZ Septra (Burroughs Wellcom)	1400 mg TMP (5 mg TMP/kg q6h for a 70-kg patient) 700 mg TMP (5 mg TMP/kg q12h for a 70-kg patient)
Erythromycin Erythromycin (Elkins-Sinn)	2000 mg (500 mg q6h)
Doxycycline Vibramycin (Pfizer)	200 mg (100 mg q12h)
Vancomycin Vancocin (Lilly)	2000 mg (500 mg q6h)
Metronidazole (generic) (Elkins-Sinn)	2000 mg (500 mg q6h)
Ciprofloxacin Cipro	200 mg q12h 400 mg q12h
Pentamidine Pentam (LyphoMed)	280 mg (4 mg/kg q24h for a 70-kg patient)

The effectiveness of erythromycin and lincomycin against a wide variety of organisms is shown in Table 9 (taken from Lorian, 1991) to illustrate the range of antibiotic resistance acquired by various bacterial strains. The data presented in the tables of the present specification is merely illustrative and is considered another tool to enable the straightforward comparison of raw data with accepted clinical practice and to allow the determination of appropriate doses of combined agents for clinical use.

TABLE 9

SUSCEPTIBILITY TO ANTIBIOTICS

ERYTHROMYCIN

Species	(n)	Range	MIC_{50}	MIC_{90}
Bacillus spp.	20	0.03-2	0.25	2
Bacteroides fragilis	97	0.25-16	1	8
Bordetella bronchiseptica	11	4-32	8	32
Bordetella parapertussis	46	0.125-4	0.25	0.25
Bordetella pertussis	32	1-0.5	0.25	0.25
Bordetella pertussis	75	0.125-0.5	0.125	0.125
Borrelia burgdorferi	10	0.03-0.125	0.03	0.06
Branhamella (Moraxella) *catarrhalis*	20	0.125-0.5	0.25	0.25
Branhamella (Moraxella) *catarrhalis*	20	0.125-0.5	0.25	1
Branhamella (Moraxella) *catarrhalis* (non β-lactamase producer)	40	0.06-0.5	0.25	0.5
Branhamella (Moraxella) *catarrhalis* (non β-lactamase producer)	13	0.03-0.125	0.06	0.06
Branhamella (Moraxella) *catarrhalis* (non β-lactamase producer)	14	0.06-1	0.125	1
Branhamella (Moraxella) *catarrhalis* (non β-lactamase producer)	16	0.015-1	0.06	0.25
Branhamella (Moraxella) *catarrhalis* (β-lactamase producer)	47	0.06-1	0.25	0.5
Branhamella (Moraxella) *catarrhalis* (β-lactamase	58	0.03-0.25	0.125	0.125

Species	(n)	Range	MIC_{50}	MIC_{90}
producer)				
Branhamella (Moraxella) *catarrhalis* (β-lactamase producer)	160	0.06-8	0.25	0.5
Branhamella (Moraxella) *catarrhalis* (β-lactamase producer)	35	0.03-0.125	0.06	0.06
Campylobacter jejuni	25	0.5-8	1	4
Campylobacter jejuni	16	0.125-4	0.25	2
Campylobacter pylori	56	0.25-16	0.5	1
Campylobacter pylori	13	0.125-0.25	0.125	0.25
Corynebacterium JK	102	0.5-128	128	128
Corynebacterium JK	19	0.125-64	2	64
Enterococcus faecalis	26	1-64	1	4
Enterococcus faecalis	50	0.06-64	4	64
Enterococcus faecalis	86	0.125-64	1	64
Enterococcus faecalis	97	0.125-128	2	128
Enterococcus faecium	14	0.06-64	1	64
Enterococcus spp.	35	0.06-32	2	32
Haemophilus ducreyi	122	?-0.125	0.004	0.06
Haemophilus influenzae	145	0.5-8	2	2
Haemophilus influenzae	97	0.25-16	1	4
Haemophilus influenzae (non β-lactamase producer)	22	0.125-8	2	4
Haemophilus influenzae (non β-lactamase producer)	137	0.06-8	4	8
Haemophilus influenzae (β-lactamase producer)	46	0.06-8	4	8
Haemophilus influenzae (β-lactamase producer)	17	0.25-4	2	4
Haemophilus influenzae (penicillin susceptible)	22	0.25-16	8	16

Species	(n)	Range	MIC_{50}	MIC_{90}
Haemophilus influenzae (penicillin resistant)	20	8-16	8	16
Haemophilus parainfluenzae	13	0.5-8	2	4
Legionella spp.	23	0.03-0.25	0.125	0.25
Legionella pneumophila	31	0.0075-0.25	0.06	0.125
Legionella pneumophila	48	0.03-2	0.25	0.5
Legionella pneumophila	25	0.125-1	0.25	1
Listeria monocytogenes	13	0.5-1	0.5	0.5
Listeria monocytogenes	16	0.125-2	0.25	1
Listeria monocytogenes	65	0.06-32	0.125	32
Mycoplasma hominis	26	128	128	128
Mycoplasma hominis	20	256	256	256
Mycoplasma pneumoniae	10	0.06-8	0.06	0.06
Mycoplasma pneumoniae	14	0.004-0.03	0.004	0.004
Neisseria gonorrhoeae	19	0.0075-8	0.25	1
Neisseria gonorrhoeae (non β-lactamase producer)	73	0.015-4	0.25	2
Neisseria gonorrhoeae (non β-lactamase producer)	78	0.03-2	0.25	1
Neisseria gonorrhoeae (β-lactamase producer)	12	0.03-4	0.5	2
Neisseria gonorrhoeae (β-lactamase producer)	17	1-4	2	4
Neisseria meningitidis	19	0.5-8	1	8
Nocardia asteroides	78	0.25-8	8	8
Staphylococcus aureus	44	0.125-1	0.125	0.5
Staphylococcus aureus	100	0.25-128	0.5	4
Staphylococcus aureus (penicillin susceptible)	20	0.125-0.5	0.5	0.5
Staphylococcus aureus (penicillin susceptible)	35	0.06-32	0.25	0.5

Species	(n)	Range	MIC_{50}	MIC_{90}
Staphylococcus aureus (penicillin resistant)	35	0.25-32	0.25	32
Staphylococcus aureus (methicillin susceptible)	28	0.125-1	0.25	0.5
Staphylococcus aureus (methicillin susceptible)	97	0.125-64	0.25	64
Staphylococcus aureus (methicillin susceptible)	20	0.125-1	0.5	0.5
Staphylococcus aureus (methicillin resistant)	17	0.5-128	128	128
Staphylococcus aureus (methicillin resistant)	15	64	64	64
Staphylococcus aureus (methicillin resistant)	20	64	64	64
Staphylococcus aureus (methicillin resistant)	30	0.06-32	32	32
Staphylococcus coagulase f	10	0.125-4	0.25	2
Staphylococcus coagulase f	100	0.125-64	0.25	64
Staphylococcus coagulase f (non β-lactamase producer)	12	0.03-8	0.125	0.25
Staphylococcus coagulase f (β-lactamase producer)	38	0.06-16	0.125	4
Staphylococcus epidermidis	50	0.125-64	64	64
Staphylococcus haemolyticus	20	0.125-64	64	64
Staphylococcus hominis	20	0.125-64	64	64
Streptococcus agalactiae	20	0.03-0.25	0.03	0.125
Streptococcus agalactiae	34	0.015-0.06	0.03	0.03
Streptococcus pneumoniae	58	0.03-0.25	0.06	0.125
Streptococcus pneumoniae	91	0.125-4	0.125	0.125
Streptococcus pneumoniae	50	0.015-0.06	0.03	0.03
Streptococcus pneumoniae	16	0.03-0.125	0.06	0.125
Streptococcus pneumoniae	26	0.015-0.25	0.03	0.06

Species	(n)	Range	MIC_{50}	MIC_{90}
Streptococcus pneumoniae	50	0.03-0.125	0.06	0.06
Streptococcus pyogenes	19	0.03-0.25	0.06	0.125
Streptococcus pyogenes	20	0.03-0.25	0.06	0.125
Streptococcus pyogenes	33	0.015-0.03	0.03	0.03
Streptococcus pyogenes	20	0.06-32	0.125	32
Streptococcus spp.	22	0.015-0.25	0.03	0.06
Streptococcus spp.	107	0.004-2	0.03	1
Ureaplasma urealyticum	28	0.015-256	2	256
Ureaplasma urealyticum	19	8-128	16	32

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Species	(n)	Range	MIC_{50}	MIC_{90}
Mycoplasma hominis	28	0.5-16	2	4
Mycoplasma pneumoniae	11	2-32	8	32
Staphylococcus aureus	100	0.5-512	1	1
Ureaplasma urealyticum	19	64-128	128	128

EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

a. BLAST-based searches

Genomic search strategies for human gene discovery were applied to the Genbank NR, HTGS and EST databases using the BLASTp and tBLASTn programs (Altschul *et al.*, 1990) using the NCBI website tools (.ncbi.nlm.nih.gov/BLAST/). Similar approaches were used to query the Celera mouse genome assembly (.celera.com). The Initial queries for the search utilized the amino acid sequences for the known human defensins (DEFB1, DEFB2, DEFB3, DEFB4) (Bensch *et al.*, 1995; Schroder *et al.*; Pend *et al.*, 2001; Harder *et al.*, 2001; Garcia *et al.*, 2001) and the EP2/HE2 sequences (Frolich *et al.*, 2000; Hamil *et al.*, 2000) and the known mouse ß-defensins (*Defb1*, *Defb2*, *Defb3*, *Defb4*, *Defb5*,*Defb6*) Huttner *et al.*, 1997; Morrison *et al.*, 1999; Bals *et al.*, 1999; Jia *et al.* 2000; Yamaguchi *et al.*, 2001) and Genbank (AF318068).

For each novel ß-defensin gene identified using the *hmmsearch* program (described below), additional iterative BLAST searches were performed against the human and mouse databases to identify additional related sequences and search for expressed sequence tags (ESTs) to confirm that the sequences are transcribed.

b. Construction of Hidden Markov Models for the six-cysteine ß-defensin motif

The complementary strategy used to identify ß-defensin genes employed a quantitative sequence analysis using the Hidden Markov Model (Eddy, 1998; Sonnhammer and Durbin, 1997; Iseli *et al.*, 1999). For this purpose, the inventors defined core human and mouse ß-defensin amino acid sequences containing the six cysteine motif and sorted them according to their scores in Hidden Markov Chain Models (HMMs) trained on *defensin* motifs. Initially, twelve 36-47 amino acid long second exon 6-cysteine motifs derived from human and mouse ß-defensin sequences previously localized to chromosomes 8p23-p22 and 8 were defined by manual inspection of full length ß-defensin domain sequences. These motifs were aligned using

the ClustalW program (Thomspon *et al.*, 1990) and trimmed of extra amino acids extending on both sides of a 33-35 amino acid core. These 12 aligned sequences were used as input for the HMMER 2.1.1 suite software (Eddy, 1998) to build the first of our HMM ß-defensin models. The program *hmmbuild* was used to construct this first model, and *hmmcalibrate* was used to calibrate E-value scores. HMMs are well-suited to this task because the scores calculated, once calibrated on the size of the data set, are directly related to the probability that the motif under consideration did not occur by chance. Furthermore, the HMM technique is more flexible and allows uncovering motif occurrences not contained in the initial training set. An optimal HMM may therefore be constructed by an iterative cycle of training and searching cycles, exploring most of the motif space.

c. Assembly of human and mouse β-defensin genomic clusters

To generate continuous DNA sequence for some analyses, the sequences from the human and mouse defensin containing BAC clones and genomic contigs, sequences were aligned using the Sequencher program (Gene Codes Corporation, Ann Arbor, MI).

d. Analysis of predicted β-defensin peptide sequences: alignment and phylogeny

The multiple sequence alignment and dendogram construction were performed using the program Pileup from the Wisconsin Package software (Accelrys, San Diego, CA). The amino acid sequences were predicted from the known, related and predicted ß-defensin genes in human and/or mouse and included two residues before and after the six-cysteine domain. The comparison matrix was set at Blosum62 with a gap creation penalty of 8 and a gap extension penalty of 2.

EXAMPLE 2

A Hidden Markov Model (HMM) (Sonnhammer *et al.*, 1997; Eddy *et al.*, 1998) was constructed with the mature peptide sequences predicted from the five known human ß-defensin genes (Bensch *et al.*, 1995; Schroder *et al.*, 1999; Harder *et al.*, 2001; Jia *et al.*, 2001; Garcia *et al.*, 2001; Frohlich *et al.*, 2000) and six mouse ß-defensin genes (Huttner *et al.*, 1997; Morrison *et al.*, 1999; Bals *et al.*, 1999; Jia *et al.*, 2000; Yamaguchi *et al*, 2001) (Genbank AF318068). The program *hmmsearch* (hmmer.wustl.edu/) used this HMM to screen about 4 Mb of genomic DNA sequence around the known ß-defensin locus on human chromosome 8p23-p22. Twelve genes were found, including the five known ß-defensin genes, DEFB1-4, and HE2/EP2, and six novel genes, DEFB4-8 and DEFBp1 (FIG. 1). When the novel sequences were used for BLAST

analysis of the human genome sequence, another ß-defensin gene was found, DEFB10. The HMM was reseeded with the predicted peptide sequence from the new genes and used to analyze the genomic DNA sequence around DEFB10. Four more ß-defensin genes, DEFB11-14, were revealed (FIG. 1). Prior to this study, all human defensin genes mapped to chromosome 8p23-p22 (Liu *et al.*, 1997; Bevins *et al.*, 1996; Harder *et al.*, 1997). Surprisingly, the DEFB10-14 genes are located on chromosome 6p12, indicating a second ß-defensin gene cluster in the human genome. The BLAST/*hmmsearch* process was iterated and 15 new ß-defensins, DEFB15-29, were found (FIG. 1). These genes are located on two sequence contigs that map to chromosome 20q11.1 and 20p13 and represent two more ß-defensin gene clusters.

Finally, the 31 human ß-defensin genes were combined in a HMM and used to analyze the six-frame translation of the entire human genome with *hmmsearch*. Two new ß-defensin genes, DEFB30 and DEFB31, were identified on the same BAC clones and represent a fifth cluster in the human genome. These genes have not been unambiguously mapped and may be located on chromosomes 2, 4, 8 or 11 (FIG. 1). Significantly, only 13 of 31 of the previously identified ß-defensin genes were detected, demonstrating that, like BLAST searches, the genome-wide searches with *hmmsearch* alone are not sufficient for identifying all ß-defensin genes. Further BLAST and *hmmsearch* analyses did not detect additional sequences in the human genome. In total, 28 novel ß-defensin genes were identified in the human genome in five clusters. The predicted partial peptide sequences for these genes are shown in FIG. 1, and the Genbank accession numbers for their genomic sequence is in Appendix 1.

To search for novel ß-defensin genes in the mouse genome, a similar approach was used to screen the mouse genome assembly in the Celera database (.celera.com). A total of 39 new sequences were found (Appendix 1) clustered on four chromosomes, 8, 1, 2 and 14. These regions of the mouse genome are syntenic to the human ß-defensin clusters at 8p23-p22, 6p12, 20p11, 20q13 and 8p23-p22 (.ncbi.nlm.nih.gov/homology). In addition, many of the predicted gene products from each human cluster were most similar to a predicted gene product located in the syntenic cluster in mouse suggesting that these genes represent homologs (FIG. 2 and Appendix 1). Finally, the order and orientation of the homologs appears to be conserved (FIG. 3). The main exceptions are the homologs between human chromosome 20 and mouse chromosome 2 where one or both clusters appears to have undergone a chromosomal rearrangement. Given the strong synteny between these five loci in the human genome and four loci in the mouse, the inventors conclude that each, individual, ß-defensin gene cluster and its syntenic partner originated from a common ancestral gene cluster (Jia *et al.*, 2000; Liu *et al.*, 1997).

To test whether these predicted genes are transcribed, the predicted amino acid sequence for each gene was queried against the six-frame translation of the expressed sequence tag database (dbEST) using tBLASTn. Sequence identity was found in dbEST for 13 human and 10 mouse predicted genes (Appendix 1). ESTs were found for at least one gene from each cluster, except for those from human 6p12/mouse 1. However, preliminary PCR expression studies using a commercially-available cDNA panel showed that all of the hypothetical genes from human 6p12 are expressed in placenta (data not shown). It is not surprising that many of the novel ß-defensin genes are not represented in the EST database. For example, the known ß-defensin gene DEFB3 is not found in the EST database. This gene is expres sed at very low levels in normal tissues, but is induced in response to inflammatory stimuli (Harder *et al.*, Jia *et al.*, 2001; Duits *et al.*, 2001). These preliminary expression studies together with the conservation of the four sequence clusters suggest that many of the 27 human and 39 mouse novel ß-defensin genes are expressed and prove that the iterative BLAST/hmmsearch method is an effective approach for gene discovery.

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All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

Appendix 1. Sequence information for human and mouse ß-defensin genes

ß-defensin Gene[a]	Chromosome	partial amino acid sequence used to build Hidden Markov Models	Accession Numbers[b] Genomic Genbank	Genomic Celera	EST
DEFB1	8p23-p22	YNCVSSGGQCLYSACPIFTKIQGTCYRGKAKCCK	NT_008268.5		AI688359
Defb1	*8*	YKCLQHGGFCLRSSCPSNTKLQGTCKPDKPNCCKS	AL590630	GA_x5J8B7W5T7M	AW226790
Defb7	*8*	TRCYKFGGFCHYNICPGNSRFMSNCHPENLRCCKN	AL590619	GA_x5J8B7W5T7M	n.d.
Defb8	*8*	ARCYKFGGFCYNSMCPPHTKFIGNCHPDHLHCCIN	AL590619	GA_x5J8B7W5T7M	AV281472
Defb2	*8*	DHCHTNGGYCVRAICPPSARRPGSCFPEKNPCCKY	AL590619	GA_x5J8B7W5T7M	AV381893
Defb9	*8*	ERCHKKGGYC-YFYCFSSHKKIGSCFPEWPRCCKN	AL590619	GA_x5J8B7W5T7M	BE991400
DEFB3	8p23-p22	YYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCCRR	NT_019483.5		n.d.
Defb14	*8*	FFCRIRGGRCAVLNCLGKEEQIGRCSNSGRKCCRK	n.d.	GA_x5J8B7W6WMR (#5)	n.d.
Defb10	*8*	VSCIRNGGIC-QYRCIGLRHKIGTCGSP-FKCCK	n.d.	GA_x5J8B7W6WMR (#6)	BG081036
Defb3	*8*	VSCLRKGGRCWNR-CIGNTRQIGSCGVPFLKCCKR	n.d.	GA_x5J8B7W6WMR (#5)	n.d.
Defb15	*8*	RACYREGGEC-LORCIGLFHKIGTC-NFRFKCCKF	n.d.	GA_x5J8B7W6WMR (#6)	n.d.
Defb4	*8*	ITCMTNGAICWGP-CPTAFRQIGNCGHFKVRCCKI	n.d.	GA_x5J8B7W6WMR (#5)	AV086680
Defb6	*8*	VTCMSYGGSC-QRSCNGSFRLGGHCGHPKIRCCRR	n.d.	GA_x5J8B7W6WMR (#5)	n.d.
Defb5	*8*	VSCCMIGGICRYL-CKGNILQNGNCGVTSLNCCKR	n.d.	GA_x5J8B7W6WMR (#5)	n.d.
DEFB2	8p23-p22	VTCLKSGAICHPVFCPRRYKQIGTCGLPGTKCCKK	NT_019483.5		bf08889
DEFB9	8p23-p22	GHCLNLSGVCRRDVCKVVEDQIGACRRRMK-CCRA	NT_019483.5		aw383156
Defb42	*14*	CVSLQGTCRRDICKLIEDEIGACRRRWK-CCRL	AC090659	GA_x5J8B7W5DQC	n.d.
DEFB30	2/4p/8p/11q	KQCIALKGVCRDKLCSTLDDTIGICNEGKK-CCRR	AC068357.2 (chr 8)		n.d.
Defb41	*14*	KQCISLKGICKDLACTSSDDTIGVCNDVKK-CCRK	AC090659	GA_x5J8B7W5DQC	n.d.
Defb38	*8*	KKCVQRKNACHYFECPWLYYSVGTCYKGKGKCCQK	n.d.	GA_x5J8B7W6WMR (#5)	n.d.
Defb40	*8*	IKCLQGNNNCHIQKCPWFLLQVSTCYKGKGRCCQK	n.d.	GA_x5J8B7W6WMR (#5)	n.d.
Defb39	*8*	IQCFQKNNTCHTNQCPYFQDEIGTCYDKRGKCCQK	n.d.	GA_x5J8B7W6WMR (#5)	n.d.
Defb37	*8*	IACIENKDTCRLKNCPRLHNVVGTCYEGKGKCCHK	n.d.	GA_x5J8B7W6WMR (#5)	n.d.
EP2d/HE2b1	8p23-p22	TICRMQQGICRLFFCHSGEKKRDICSDPWNRCCVS	NT_019483.5		aa778602
Ep2d	*8*	TVCLMQQGHCRLFMCRSGERKGDICSDPWNRCCVP	n.d.	GA_x5J8B7W6WMR (#5)	n.d.
DEFB31	2/4p/8p/11q	DECPSEYYHCRLK-CNADEHAIRYCADFSI-CCKL	AC068357.2 (chr 8)		n.d.
Defb43	*14*	QDCSKHRH-CRMK-CKANEYAVRYCEDWTI-CCRV	AC090659	GA_x5J8B7W5DQC	n.d.
DEFB26 (ESP13.2)	20p13	KKCLNDVGICKKK-CKPEEMHVKNGWAMCGKQRDCCV	NT_011493.5	GA_x5L2HTTBG3J	AA994981
Defb22	*2*	KKCANTLGNCRKM-CRDGEKQTEPATSKCPIGKLCCV	n.d.	GA_x5J8B7W3FJ8	n.d.
DEFB29	20p13	RRCLMGLGRCRDH-CNVDEKEIQKCKMKK--CCVG	NT_011493.5	GA_x5L2HTTBG3J	AA401404
Defb23	*2*	KRCLVGFGKCKDS-CLADETQMQHCKAKK--CCIG	n.d.	GA_x5J8B7W3FJ8	BE646673

DEFB15	20q11.1	RRCYYGTGRCRK-SCKEIERKKEKCGEKHI-CCVP	NT_028392.2	GA_x5L2HTTW9JV	n.d.
Defb28	*2*	RTCFYGLGKCRR-ICRANEKKKERC-GERTFCCLR	n.d.	GA_x5J8B7W22L0	AV044615
DEFB4	8p23-p22	RICGYGTARCRKK-CRSQEYRIGRCPNTYA-CCLR	NT_019483.5		n.d.
DEFB16	20q11.1	NPCELYQGMCRNA-CREYEIQYLTCPNDQK-CCLK	NT_028392.2	GA_x5L2HTTW9JV	n.d.
Defb29	*2*	IACELYQGLC-RNACQKYEIQYLSC-PKTRKCCLK	n.d.	GA_x5J8B7W22L0	AI552035
DEFB19	20q11.1	LRCMGNSGICRAS-CKKNEQPYLYCRNCQS-CCLQ	NT_028392.2	GA_x5L2HTTW9JV	AA939044
Defb24	*2*	LQCMGNRGFC-RSSCKKSEQAYFYCRTFQM-CCLQ	n.d.	GA_x5J8B7W3FJ8	AV043850
DEFB28	20p13	KKCFNKVTGYCRKKCKVGERYEIGCLSGKL-CCAN	NT_011493.5	GA_x5L2HTTBG3J	n.d.
Defb20	*2*	KRCFSNVEGYCRKKCRLVEISEMGC-LHGKYCC	n.d.	GA_x5J8B7W3FJ8	AW045275
DEFB27	20p13	KKCWNNYVQGHCRKICRVNEVPEALCENGRYCCLN	NT_011493.5	GA_x5L2HTTBG3J	AI694319
DEFB17	20q11.1	KSCWIIKGHCRKNCKPGEQVKKP-CKNGDY-CCIP	NT_028392.2	GA_x5L2HTTW9JV	n.d.
Defb19	*2*	KACWVLRGHC-RKHCRSGERVRKPC-SNGDYCC	n.d.	GA_x5J8B7W3FJ8	n.d.
DEFB18	20q11.1	KKCWNRSGHCRKQ-CKDGEAVKDTCKNLRA-CCIP	NT_028392.2	GA_x5L2HTTW9JV	AA335178
Defb21	*2*	KRCLKILGHC-RRHCKDGEMDHGSC-KYYRVCCVP	n.d.	GA_x5J8B7W3FJ8	n.d.
DEFB20	20q11.1	VECWMDGH-CRLL-CKDGEDSIIRCRNRKR-CCVP	NT_028392.2	GA_x5L2HTTW9JV	AW070283
DEFB25	20p13	QKCWKNNVGHCRRRCLDTERYILLCRNKLS-CCIS	NT_011493.5	GA_x5L2HTTBG3J	AA935636
Defb26	*2*	KCWKNSLGYCRVRCQEEERYIYLCKNKVS-CCIH	n.d.	GA_x5J8B7W3FJ8	n.d.
DEFB24	20q11.1	KRCWKGQGACQTY-CTRQETYMHLCPDASL-CCLS	NT_028392.2	GA_x5L2HTTW9JV	n.d.
Defb25	*2*	KRCWNGQGAC-RTFCTRQETFMHLCPDASL-CCLS	n.d.	GA_x5J8B7W3FJ8	n.d.
DEFB23	20q11.1	QRCWNLYGKCRYR-CSKKERVYVYCINNKM-CCVK	NT_028392.2	GA_x5L2HTTW9JV	AA933749
Defb36	*U*	QKCWNLHGKC-RHRCSRKESVYVYCTNGKM-CCVK	n.d.		n.d.
Defb27	*2*	ERCWKSFGVC-REECAKKESFYIFCWNGKL-CCVK	n.d.	GA_x5J8B7W3FJ8	AI415386
DEFB22	20q11.1	ETCWNFRGSCRDE-CLKNERVYVFCVSGKL-CCLK	NT_028392.2	GA_x5L2HTTW9JV	AI989655
DEFB21	20q11.1	MKCWGKSGRCRTT-CKESEVYYILCKTEAK-CCVD	NT_028392.2	GA_x5L2HTTW9JV	AI476463
Defb30	*14*	DTCWKLKGIC-RNTCQKEEIYHIFCGIQSL-CCLE	AC090659	GA_x5J8B7W5DQC	n.d.
DEFB11	6p21	RECRIGNGQCKNQ-CHENEIRIAYCIRPGTHCCLQ	NT_007402.5	GA_x54KREAYBCL	n.d.
Defb17	*1*	KECKMRRGHC-KLQCSEKELRISFCIRPGTHCC	n.d.	GA_x5J8B7W3NRM	n.d.
DEFB12	6p21	KSCTAIGGRCKNQ-CDDSEFRISYCARPTTHCCVT	NT_007402.5	GA_x54KREAYBCL	n.d.
DEFB14	6p21	DRCTKRYGRCKRD-CLESEKQIDICSLPRKICCTE	NT_007402.5	GA_x54KREAYBCL	n.d.
EP2c	8p23-p22	VDCRRSEGFCQEY-CNYMETQVGYCSKKKDACCLH	NT_019483.5		AA778602
Ep2c	*8*	VNCKKSEGQCQEY-CNFMETQVGYCSKKKEPCCLH	n.d.	GA_x5J8B7W6WMR (#5)	n.d.
DEFB10	6p21	ERCEKVRGICKTF-CDDVEYDYGYCIKWRSQCCV	NT_007402.5	GA_x54KREAYBCL	n.d.
Defb16	*1*	ERCEKVRGMC-KTVCDIDEYDYGYCIRWRNQCCI	n.d.	GA_x5J8B7W3NRM	n.d.
DEFB13	6p21	KRECQLVRGACKPECNSWEYVYYYCNVNP--CCAV	NT_007402.5	GA_x54KREAYBCL	n.d.
Defb18	*1*	HKCSLVRGTC-KSECNSWEYKYNYC--HTEPCCVV	n.d.	GA_x5J8B7W3NRM	n.d.
Defb12	*8*	ETCRLGRGKCRRT-CIESEKIAGWCKLNFF-CCRE	n.d.	GA_x5J8B7W6WMR (#5)	n.d.
Defb35	*8*	ETCRLGRGKC-RRACIESEKIVGWCKLNFF-CCRE	AL590619	GA_x5J8B7W5T7M	n.d.

DEFB5	8p23-p22	ESCKLGRGKCRKE-CLENEKPDGNCRLNFL-CCRQ	NT_019483.5		n.d.
DEFB7	8p23-p22	TNCFLYLARTAIHRALISKRMEGHCEAE-CLTFEVKI	NT_019483.5		n.d.
Defb13	*8*	FLCKKMNGQC-EAECFTFEQKIGTC-QANFLCCRK	AL590619	GA_x5J8B7W5T7M	n.d.
Defb11	*8*	EKCSRVNGRCTAS-CLKNEELVALC-QKNLKCCVT	AL590619	GA_x5J8B7W5T7M	n.d.
Defb34	*8*	EKCSRINGRC-TASCLKNEELVALCWKNLK-CCVT	n.d.	GA_x5J8B7W6WMR (#5)	n.d.
DEFB6	8p23-p22	EKCNKLKGTCKNN-CGKNEELIALCQKSLK-CCRT	NT_019483.5		aw103145
DEFB8	8p23-p22	EICERPNGSCRDF-CLETEIHVGRCLNSRP-CCLP	NT_019483.5		aa406058
Defb32	*U*	KLCLDQKDTCPDSRTC-LEGTQP-CHPHHPNCCES	n.d.	GA_x5J8B7W66LW	n.d.
Defb33	*U*	RPCEKMGGICKSQKTHGCSILPAECKSRYKHCCRL	n.d.	GA_x5J8B7W3LE6	n.d.
Defb31	*U*	CRSWGTCSIAAICFDSLSRRGQCGPVKDPCCPL	n.d.	GA_x5J8B7W72BC	BG968591
DEFBp1	8p23-p22	ZRCVCVLNVCSTSLKQIGTYGHDRIKCCKK	NT_019483.5		pseudogene
Defbp1	*8*	LTCIANRGFC-WHSCIQGFQLAGHCGHPKVRLLH	n.d.	GA_x5J8B7W6WMR (#5)	pseudogene
Defbp2	*8*	LVCRRKGGRC-YIKCPDNTDZIGMCRLP-FKCCKRQ	n.d.	A_x5J8B7W6WMR (#6)	pseudogene
Defbp3	*U*	LSCWMKZGIC-QYRCFGNTHKIGSCGAPFLKCCKR	n.d.	GA_x5J8B7W3LE6	pseudogene

[a] Human genes are capitalized and mouse genes are in italics.

[b] No data (n.d.). Only a single accession number is given for each gene, though others may exist.

WO 03/024992 PCT/US02/30106

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CLAIMS

1. An isolated antimicrobial peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOS:1-82.

2. The antimicrobial peptide of claim 1, wherein said antimicrobial peptide is comprised in a pharmaceutically acceptable composition.

3. The antimicrobial peptide of claim 2, wherein said pharmaceutical composition is formulated for topical administration.

4. The antimicrobial peptide of claim 2, wherein said pharmaceutical composition is formulated for oral administration.

5. The antimicrobial peptide of claim 2, wherein said pharmaceutical composition is formulated for parenteral administration.

6. The antimicrobial peptide of claim 5, wherein said pharmaceutical composition is formulated for administration by injection.

7. The antimicrobial peptide of claim 5, wherein said pharmaceutical composition is formulated for administration by inhalation.

8. An isolated nucleic acid molecule encoding a peptide selected from the group consisting of SEQ ID NOS:1-82, said nucleic acid molecule isolated free from other human or murine coding sequences.

9. The nucleic acid molecule of claim 8, wherein said nucleic acid is incorporated into an expression vector.

10. A viral vector comprising a nucleic acid molecule encoding a peptide selected from the group consisting of SEQ ID NOS:1-82.

11. The viral vector of claim 10, wherein said viral vector is selected from the group consisting of adenovirus, adeno-associated virus, vaccinia virus, retrovirus, herpesvirus, and polyomavirus.

12. An isolated nucleic acid molecule encoding a peptide selected from the group consisting of SEQ ID NOS:1-82, and a promoter heterologous to the coding region for said peptide.

13. The isolated nucleic acid molecule of claim 12, wherein said promoter is CMV IE.

14. The isolated nucleic acid molecule of claim 12, further comprising one or more of an origin of replication, a polyadenylation signal, an internal ribosome entry site, a multipurpose cloning site and a selectable marker.

15. An isolated nucleic acid molecule encoding a peptide selected from the group consisting of SEQ ID NOS:1-82, said nucleic acid molecule being 10,000 base pair in length or shorter.

16. The isolated nucleic acid molecule of claim 15, said nucleic acid molecule being 5000 base pairs or shorter.

17. The isolated nucleic acid molecule of claim 15, said nucleic acid molecule being 2500 base pairs or shorter.

18. The isolated nucleic acid molecule of claim 15, said nucleic acid molecule being 1000 base pairs or shorter.

19. The isolated nucleic acid molecule of claim 15, said nucleic acid molecule being 500 base pairs or shorter.

20. A method of inhibiting the growth of a microbe comprising introducing into an environment containing said microbe a peptide selected from the group consisting of SEQ ID NOS:1-82.

21. The method of claim 20, wherein said peptide is introduced in a composition capable of sustaining the antimicrobial properties of said peptide in said environment.

22. The method of claim 21, wherein said peptide is delivered in a pharmaceutical composition.

23. The method of claim 20, further comprising introducing an additional antimicrobial agent into said environment.

24. The method of claim 23, wherein said peptide is introduced before said additional antimicrobial agent.

25. The method of claim 23, wherein said peptide and said additional antimicrobial agent are introduced concurrently.

26. The method of claim 23, wherein said peptide is introduced after said additional antimicrobial agent.

27. The method of claim 23, wherein said additional antimicrobial agent is selected from the group consisting of a protein synthesis inhibitor, a cell wall growth inhibitor, a cell membrane synthesis inhibitor, a nucleic acid synthesis inhibitor, and a competitive inhibitor.

28. The method of claim 20, wherein said environment is a surgical field or wound site.

29. A kit comprising an antimicrobial peptide, wherein said peptide comprises an amino acid sequence selected from the group consisting of SEQ ID NOS:1-82, disposed in a suitable container.

30. The kit of claim 29, further comprising an additional antimicrobial agent.

31. A method of inhibiting growth of a microbe in a host, comprising administering to said host a peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOS:1-82.

32. The method of claim 31, further comprising administering an additional antimicrobial agent.

33. The method of claim 32, wherein said peptide is administered before said additional antimicrobial agent.

34. The method of claim 32, wherein said peptide and said additional antimicrobial agent are administered concurrently.

35. The method of claim 32, wherein said peptide is administered after said additional antimicrobial agent.

36. The method of claim 32, wherein said additional antimicrobial agent is selected from the group consisting of a protein synthesis inhibitor, a cell wall growth inhibitor, a cell membrane synthesis inhibitor, a nucleic acid synthesis inhibitor, and a competitive inhibitor.

37. A medical device coated with one or more peptides selected from the group consisting of SEQ ID NOS:1-82.

38. The medical device of claim 37, wherein said medical device is a catheter, a needle, a sheath, and a stent.

39. An antimicrobial composition comprising one or more peptides selected from the group consisting of SEQ ID NOS:1-82 and one or more non-peptide antimicrobial agents.

40. A method of treating a bacterial infection comprising administering to a subject a peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOS:1-82.

41. A method of activating a memory T cell comprising contacting a memory T cell with a peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOS:1-82.

42. A method of activating an immature dendritic cell comprising contacting an immature dendritic cell with a peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOS:1-82.

43. A method of stimulating adaptive immune response comprising contacting a subject with a peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOS:1-82.

44. A method of inhibiting a multidrug resistant bacterium comprising treating said bacterium with a peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOS:1-82.

45. The method of claim 44, further comprising treating said bacterium with an additional antimicrobial agent.

Gene	Chr	Gene class	Amino acid sequence of six-cysteine domain
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DEFB03(ref)	8	K	YYCRV--RGGRCAVLSCLPKEEQIGKCST-RGRKCCRRKK
DEFB04(ref)	8	K	RICGY--GTARCRKK-CRSQEYRIGRCPN--TYACCLRK
DEFB05	8	P	ESCKL--GRGKCRKE-CLENEKPDGNCRL--NFLCCRQRI
DEFB06	8	R	EKCNK--LKGTCKNN-CGKNEELIALCQK--SLKCCRTIQPCGSIID
DEFB07	8	P	TNC (+21) CEAE-CLTFEVKIGGCRAELAPFCCKNRKKH
DEFB08	8	P	EICER--PNGSCRDF-CLETEIHVGRCLN--SRPCCLPLGHQ+11
DEFB09	8	P	GHCLN--LSGVCRRDVCKVVEDQIGACRR--RMKCCRAWWIL+21
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EP2d	8	K	TICRM--QQGICRLFFCHSGEKKRDICSD-PWNRCCVSNTDE
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FIG. 1

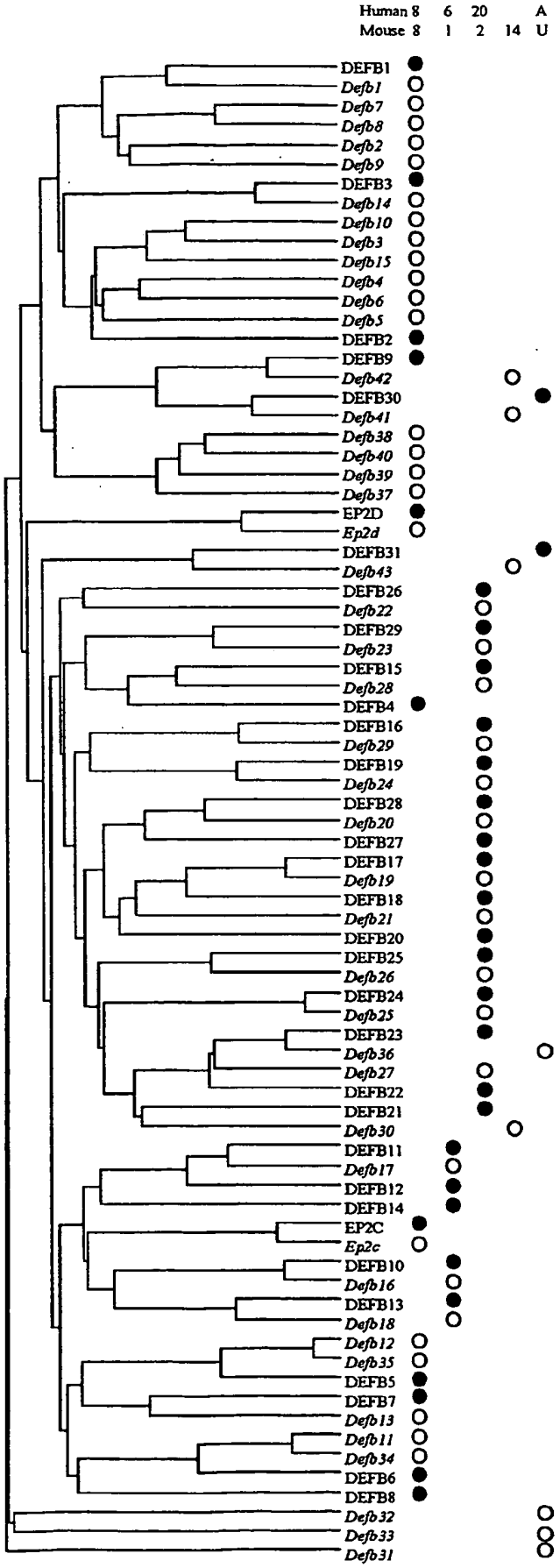
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FIG. 2

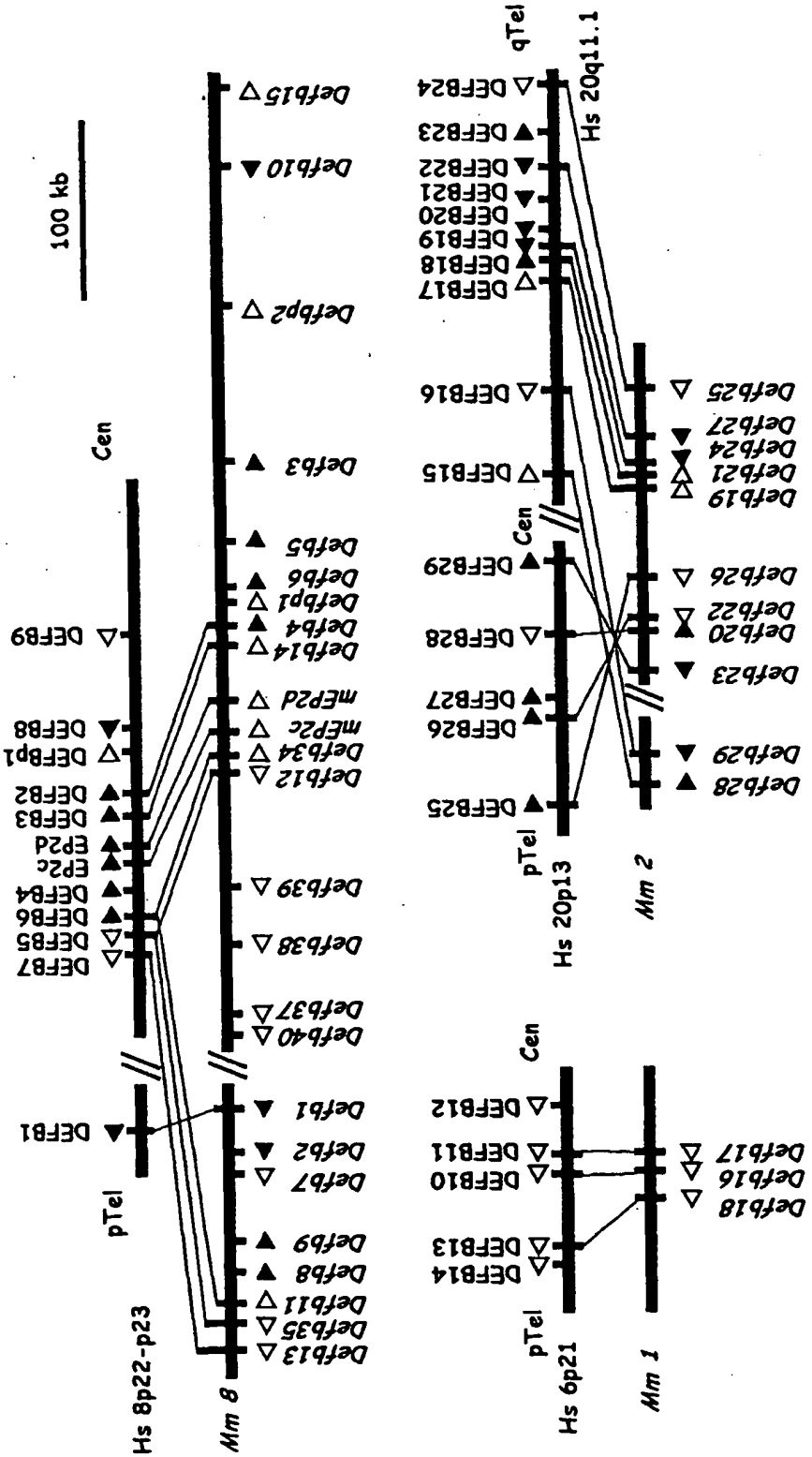
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FIG. 3

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SCHUTTE, BRIAN C.
JIA, HONG PENG
CASAVANT, THOMAS L.

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<212> PRT
<213> Homo sapiens

<400> 28
Lys Lys Cys Leu Asn Asp Val Gly Ile Cys Lys Lys Lys Cys Lys Pro
1 5 10 15

Glu Glu Met His Val Lys Asn Gly Trp Ala Met Cys Gly Lys Gln Arg
20 25 30

Asp Cys Cys Val Pro
35

<210> 29
<211> 37
<212> PRT
<213> Mus musculus

<400> 29
Lys Lys Cys Ala Asn Thr Leu Gly Asn Cys Arg Lys Met Cys Arg Asp
1 5 10 15

Gly Glu Lys Gln Thr Glu Pro Ala Thr Ser Lys Cys Pro Ile Gly Lys

20 25 30

Leu Cys Cys Val Leu
35

<210> 30
<211> 32
<212> PRT
<213> Homo sapiens

<400> 30
Arg Arg Cys Leu Met Gly Leu Gly Arg Cys Arg Asp His Cys Asn Val
1 5 10 15

Asp Glu Lys Glu Ile Gln Lys Cys Lys Met Lys Lys Cys Cys Val Gly
20 25 30

<210> 31
<211> 32
<212> PRT
<213> Mus musculus

<400> 31
Lys Arg Cys Leu Val Gly Phe Gly Lys Cys Lys Asp Ser Cys Leu Ala
1 5 10 15

Asp Glu Thr Gln Met Gln His Cys Lys Ala Lys Lys Cys Cys Ile Gly
20 25 30

<210> 32
<211> 33
<212> PRT
<213> Homo sapiens

<400> 32
Arg Arg Cys Tyr Tyr Gly Thr Gly Arg Cys Arg Lys Ser Cys Lys Glu
1 5 10 15

Ile Glu Arg Lys Lys Glu Lys Cys Gly Glu Lys His Ile Cys Cys Val
20 25 30

Pro

<210> 33
<211> 33
<212> PRT
<213> Mus musculus

<400> 33

Arg Thr Cys Phe Tyr Gly Leu Gly Lys Cys Arg Arg Ile Cys Arg Ala
1 5 10 15

Asn Glu Lys Lys Lys Glu Arg Cys Gly Glu Arg Thr Phe Cys Cys Leu
20 25 30

Arg

<210> 34
<211> 33
<212> PRT
<213> Homo sapiens

<400> 34
Arg Ile Cys Gly Tyr Gly Thr Ala Arg Cys Arg Lys Lys Cys Arg Ser
1 5 10 15

Gln Glu Tyr Arg Ile Gly Arg Cys Pro Asn Thr Tyr Ala Cys Cys Leu
20 25 30

Arg

<210> 35
<211> 33
<212> PRT
<213> Homo sapiens

<400> 35
Asn Pro Cys Glu Leu Tyr Gln Gly Met Cys Arg Asn Ala Cys Arg Glu
1 5 10 15

Tyr Glu Ile Gln Tyr Leu Thr Cys Pro Asn Asp Gln Lys Cys Cys Leu
20 25 30

Lys

<210> 36
<211> 33
<212> PRT
<213> Mus musculus

<400> 36
Ile Ala Cys Glu Leu Tyr Gln Gly Leu Cys Arg Asn Ala Cys Gln Lys
1 5 10 15

Tyr Glu Ile Gln Tyr Leu Ser Cys Pro Lys Thr Arg Lys Cys Cys Leu
20 25 30

Lys

<210> 37
<211> 33

<212> PRT
<213> Homo sapiens

<400> 37
Leu Arg Cys Met Gly Asn Ser Gly Ile Cys Arg Ala Ser Cys Lys Lys
1 5 10 15

Asn Glu Gln Pro Tyr Leu Tyr Cys Arg Asn Cys Gln Ser Cys Cys Leu
20 25 30

Gln

<210> 38
<211> 33
<212> PRT
<213> Mus musculus

<400> 38
Leu Gln Cys Met Gly Asn Arg Gly Phe Cys Arg Ser Ser Cys Lys Lys
1 5 10 15

Ser Glu Gln Ala Tyr Phe Tyr Cys Arg Thr Phe Gln Met Cys Cys Leu
20 25 30

Gln

<210> 39
<211> 34
<212> PRT
<213> Homo sapiens

<400> 39
Lys Lys Cys Phe Asn Lys Val Thr Gly Tyr Cys Arg Lys Lys Cys Lys
1 5 10 15

Val Gly Glu Arg Tyr Glu Ile Gly Cys Leu Ser Gly Lys Leu Cys Cys
20 25 30

Ala Asn

<210> 40
<211> 32
<212> PRT
<213> Mus musculus

<400> 40
Lys Arg Cys Phe Ser Asn Val Glu Gly Tyr Cys Arg Lys Lys Cys Arg
1 5 10 15

Leu Val Glu Ile Ser Glu Met Gly Cys Leu His Gly Lys Tyr Cys Cys
20 25 30

<210> 41
<211> 35
<212> PRT
<213> Homo sapiens

<400> 41
Lys Lys Cys Trp Asn Asn Tyr Val Gln Gly His Cys Arg Lys Ile Cys
1 5 10 15

Arg Val Asn Glu Val Pro Glu Ala Leu Cys Glu Asn Gly Arg Tyr Cys
20 25 30

Cys Leu Asn
35

<210> 42
<211> 33
<212> PRT
<213> Homo sapiens

<400> 42
Lys Ser Cys Trp Ile Ile Lys Gly His Cys Arg Lys Asn Cys Lys Pro
1 5 10 15

Gly Glu Gln Val Lys Lys Pro Cys Lys Asn Gly Asp Tyr Cys Cys Ile
20 25 30

Pro

<210> 43
<211> 31
<212> PRT
<213> Mus musculus

<400> 43
Lys Ala Cys Trp Val Leu Arg Gly His Cys Arg Lys His Cys Arg Ser
1 5 10 15

Gly Glu Arg Val Arg Lys Pro Cys Ser Asn Gly Asp Tyr Cys Cys
20 25 30

<210> 44
<211> 33
<212> PRT
<213> Homo sapiens

<400> 44
Lys Lys Cys Trp Asn Arg Ser Gly His Cys Arg Lys Gln Cys Lys Asp
1 5 10 15

Gly Glu Ala Val Lys Asp Thr Cys Lys Asn Leu Arg Ala Cys Cys Ile
20 25 30

Pro

<210> 45
<211> 33
<212> PRT
<213> Mus musculus

<400> 45
Lys Arg Cys Leu Lys Ile Leu Gly His Cys Arg Arg His Cys Lys Asp
1 5 10 15

Gly Glu Met Asp His Gly Ser Cys Lys Tyr Tyr Arg Val Cys Cys Val
20 25 30

Pro

<210> 46
<211> 32
<212> PRT
<213> Homo sapiens

<400> 46
Val Glu Cys Trp Met Asp Gly His Cys Arg Leu Leu Cys Lys Asp Gly
1 5 10 15

Glu Asp Ser Ile Ile Arg Cys Arg Asn Arg Lys Arg Cys Cys Val Pro
20 25 30

<210> 47
<211> 34
<212> PRT
<213> Homo sapiens

<400> 47
Gln Lys Cys Trp Lys Asn Asn Val Gly His Cys Arg Arg Arg Cys Leu
1 5 10 15

Asp Thr Glu Arg Tyr Ile Leu Leu Cys Arg Asn Lys Leu Ser Cys Cys
20 25 30

Ile Ser

<210> 48
<211> 33
<212> PRT
<213> Mus musculus

<400> 48
Lys Cys Trp Lys Asn Ser Leu Gly Tyr Cys Arg Val Arg Cys Gln Glu
1 5 10 15

Glu Glu Arg Tyr Ile Tyr Leu Cys Lys Asn Lys Val Ser Cys Cys Ile
20 25 30

His

<210> 49
<211> 33
<212> PRT
<213> Homo sapiens

<400> 49
Lys Arg Cys Trp Lys Gly Gln Gly Ala Cys Gln Thr Tyr Cys Thr Arg
1 5 10 15

Gln Glu Thr Tyr Met His Leu Cys Pro Asp Ala Ser Leu Cys Cys Leu
20 25 30

Ser

<210> 50
<211> 33
<212> PRT
<213> Mus musculus

<400> 50
Lys Arg Cys Trp Asn Gly Gln Gly Ala Cys Arg Thr Phe Cys Thr Arg
1 5 10 15

Gln Glu Thr Phe Met His Leu Cys Pro Asp Ala Ser Leu Cys Cys Leu
20 25 30

Ser

<210> 51
<211> 33
<212> PRT
<213> Homo sapiens

<400> 51
Gln Arg Cys Trp Asn Leu Tyr Gly Lys Cys Arg Tyr Arg Cys Ser Lys
1 5 10 15

Lys Glu Arg Val Tyr Val Tyr Cys Ile Asn Asn Lys Met Cys Cys Val
20 25 30

Lys

<210> 52
<211> 33
<212> PRT
<213> Mus musculus

<400> 52
Gln Lys Cys Trp Asn Leu His Gly Lys Cys Arg His Arg Cys Ser Arg
1 5 10 15

Lys Glu Ser Val Tyr Val Tyr Cys Thr Asn Gly Lys Met Cys Cys Val
20 25 30

Lys

<210> 53
<211> 33
<212> PRT
<213> Mus musculus

<400> 53
Glu Arg Cys Trp Lys Ser Phe Gly Val Cys Arg Glu Glu Cys Ala Lys
1 5 10 15

Lys Glu Ser Phe Tyr Ile Phe Cys Trp Asn Gly Lys Leu Cys Cys Val
20 25 30

Lys

<210> 54
<211> 33
<212> PRT
<213> Mus musculus

<400> 54
Glu Thr Cys Trp Asn Phe Arg Gly Ser Cys Arg Asp Glu Cys Leu Lys
1 5 10 15

Asn Glu Arg Val Tyr Val Phe Cys Val Ser Gly Lys Leu Cys Cys Leu
20 25 30

Lys

<210> 55
<211> 33
<212> PRT
<213> Mus musculus

<400> 55
Met Lys Cys Trp Gly Lys Ser Gly Arg Cys Arg Thr Thr Cys Lys Glu
1 5 10 15

Ser Glu Val Tyr Tyr Ile Leu Cys Lys Thr Glu Ala Lys Cys Cys Val
20 25 30

Asp

<210> 56

<211> 33
<212> PRT
<213> Mus musculus

<400> 56
Asp Thr Cys Trp Lys Leu Lys Gly Ile Cys Arg Asn Thr Cys Gln Lys
1 5 10 15

Glu Glu Ile Tyr His Ile Phe Cys Gly Ile Gln Ser Leu Cys Cys Leu
20 25 30

Glu

<210> 57
<211> 34
<212> PRT
<213> Mus musculus

<400> 57
Arg Glu Cys Arg Ile Gly Asn Gly Gln Cys Lys Asn Gln Cys His Glu
1 5 10 15

Asn Glu Ile Arg Ile Ala Tyr Cys Ile Arg Pro Gly Thr His Cys Cys
20 25 30

Leu Gln

<210> 58
<211> 32
<212> PRT
<213> Mus musculus

<400> 58
Lys Glu Cys Lys Met Arg Arg Gly His Cys Lys Leu Gln Cys Ser Glu
1 5 10 15

Lys Glu Leu Arg Ile Ser Phe Cys Ile Arg Pro Gly Thr His Cys Cys
20 25 30

<210> 59
<211> 34
<212> PRT
<213> Mus musculus

<400> 59
Lys Ser Cys Thr Ala Ile Gly Gly Arg Cys Lys Asn Gln Cys Asp Asp
1 5 10 15

Ser Glu Phe Arg Ile Ser Tyr Cys Ala Arg Pro Thr Thr His Cys Cys
20 25 30

Val Thr

<210> 60
<211> 34
<212> PRT
<213> Mus musculus

<400> 60
Asp Arg Cys Thr Lys Arg Tyr Gly Arg Cys Lys Arg Asp Cys Leu Glu
1 5 10 15

Ser Glu Lys Gln Ile Asp Ile Cys Ser Leu Pro Arg Lys Ile Cys Cys
20 25 30

Thr Glu

<210> 61
<211> 34
<212> PRT
<213> Mus musculus

<400> 61
Val Asp Cys Arg Arg Ser Glu Gly Phe Cys Gln Glu Tyr Cys Asn Tyr
1 5 10 15

Met Glu Thr Gln Val Gly Tyr Cys Ser Lys Lys Lys Asp Ala Cys Cys
20 25 30

Leu His

<210> 62
<211> 34
<212> PRT
<213> Mus musculus

<400> 62
Val Asn Cys Lys Lys Ser Glu Gly Gln Cys Gln Glu Tyr Cys Asn Phe
1 5 10 15

Met Glu Thr Gln Val Gly Tyr Cys Ser Lys Lys Lys Glu Pro Cys Cys
20 25 30

Leu His

<210> 63
<211> 33
<212> PRT
<213> Mus musculus

<400> 63
Glu Arg Cys Glu Lys Val Arg Gly Ile Cys Lys Thr Phe Cys Asp Asp
1 5 10 15

Val Glu Tyr Asp Tyr Gly Tyr Cys Ile Lys Trp Arg Ser Gln Cys Cys
20 25 30

Val

<210> 64
<211> 33
<212> PRT
<213> Mus musculus

<400> 64
Glu Arg Cys Glu Lys Val Arg Gly Met Cys Lys Thr Val Cys Asp Ile
1 5 10 15

Asp Glu Tyr Asp Tyr Gly Tyr Cys Ile Arg Trp Arg Asn Gln Cys Cys
20 25 30

Ile

<210> 65
<211> 33
<212> PRT
<213> Mus musculus

<400> 65
Lys Arg Glu Cys Gln Leu Val Arg Gly Ala Cys Lys Pro Glu Cys Asn
1 5 10 15

Ser Trp Glu Tyr Val Tyr Tyr Tyr Cys Asn Val Asn Pro Cys Cys Ala
20 25 30

Val

<210> 66
<211> 32
<212> PRT
<213> Mus musculus

<400> 66
His Lys Cys Ser Leu Val Arg Gly Thr Cys Lys Ser Glu Cys Asn Ser
1 5 10 15

Trp Glu Tyr Lys Tyr Asn Tyr Cys His Thr Glu Pro Cys Cys Val Val
20 25 30

<210> 67
<211> 33
<212> PRT
<213> Mus musculus

<400> 67
Glu Thr Cys Arg Leu Gly Arg Gly Lys Cys Arg Arg Thr Cys Ile Glu
1 5 10 15

Ser Glu Lys Ile Ala Gly Trp Cys Lys Leu Asn Phe Phe Cys Cys Arg
20 25 30

Glu

<210> 68
<211> 33
<212> PRT
<213> Mus musculus

<400> 68
Glu Thr Cys Arg Leu Gly Arg Gly Lys Cys Arg Arg Ala Cys Ile Glu
1 5 10 15

Ser Glu Lys Ile Val Gly Trp Cys Lys Leu Asn Phe Phe Cys Cys Arg
20 25 30

Glu

<210> 69
<211> 33
<212> PRT
<213> Mus musculus

<400> 69
Glu Ser Cys Lys Leu Gly Arg Gly Lys Cys Arg Lys Glu Cys Leu Glu
1 5 10 15

Asn Glu Lys Pro Asp Gly Asn Cys Arg Leu Asn Phe Leu Cys Cys Arg
20 25 30

Gln

<210> 70
<211> 50
<212> PRT
<213> Mus musculus

<400> 70
Thr Asn Cys Phe Leu Tyr Leu Ala Arg Thr Ala Ile His Arg Ala Leu
1 5 10 15

Ile Ser Lys Arg Met Glu Gly His Cys Glu Ala Glu Cys Leu Thr Phe
20 25 30

Glu Val Lys Ile Gly Gly Cys Arg Ala Glu Leu Ala Pro Phe Cys Cys
35 40 45

Lys Asn
50

<210> 71
<211> 33
<212> PRT
<213> Mus musculus

<400> 71
Phe Leu Cys Lys Lys Met Asn Gly Gln Cys Glu Ala Glu Cys Phe Thr
1 5 10 15

Phe Glu Gln Lys Ile Gly Thr Cys Gln Ala Asn Phe Leu Cys Cys Arg
20 25 30

Lys

<210> 72
<211> 33
<212> PRT
<213> Mus musculus

<400> 72
Glu Lys Cys Ser Arg Val Asn Gly Arg Cys Thr Ala Ser Cys Leu Lys
1 5 10 15

Asn Glu Glu Leu Val Ala Leu Cys Gln Lys Asn Leu Lys Cys Cys Val
20 25 30

Thr

<210> 73
<211> 33
<212> PRT
<213> Mus musculus

<400> 73
Glu Lys Cys Ser Arg Ile Asn Gly Arg Cys Thr Ala Ser Cys Leu Lys
1 5 10 15

Asn Glu Glu Leu Val Ala Leu Cys Trp Lys Asn Leu Lys Cys Cys Val
20 25 30

Thr

<210> 74
<211> 33
<212> PRT
<213> Mus musculus

<400> 74
Glu Lys Cys Asn Lys Leu Lys Gly Thr Cys Lys Asn Asn Cys Gly Lys
1 5 10 15

Asn Glu Glu Leu Ile Ala Leu Cys Gln Lys Ser Leu Lys Cys Cys Arg

20 25 30

Thr

<210> 75
<211> 33
<212> PRT
<213> Mus musculus

<400> 75
Glu Ile Cys Glu Arg Pro Asn Gly Ser Cys Arg Asp Phe Cys Leu Glu
1 5 10 15

Thr Glu Ile His Val Gly Arg Cys Leu Asn Ser Arg Pro Cys Cys Leu
20 25 30

Pro

<210> 76
<211> 33
<212> PRT
<213> Mus musculus

<400> 76
Lys Leu Cys Leu Asp Gln Lys Asp Thr Cys Pro Asp Ser Arg Thr Cys
1 5 10 15

Leu Glu Gly Thr Gln Pro Cys His Pro His His Pro Asn Cys Cys Glu
20 25 30

Ser

<210> 77
<211> 35
<212> PRT
<213> Mus musculus

<400> 77
Arg Pro Cys Glu Lys Met Gly Gly Ile Cys Lys Ser Gln Lys Thr His
1 5 10 15

Gly Cys Ser Ile Leu Pro Ala Glu Cys Lys Ser Arg Tyr Lys His Cys
20 25 30

Cys Arg Leu
35

<210> 78
<211> 33
<212> PRT
<213> Mus musculus

<400> 78

Cys Arg Ser Trp Gly Thr Cys Ser Ile Ala Ala Ile Cys Phe Asp Ser
1 5 10 15

Leu Ser Arg Arg Gly Gln Cys Gly Pro Val Lys Asp Pro Cys Cys Pro
20 25 30

Leu

<210> 79
<211> 33
<212> PRT
<213> Mus musculus

<400> 79
Cys Arg Ser Trp Gly Thr Cys Ser Ile Ala Ala Ile Cys Phe Asp Ser
1 5 10 15

Leu Ser Arg Arg Gly Gln Cys Gly Pro Val Lys Asp Pro Cys Cys Pro
20 25 30

Leu

<210> 80
<211> 33
<212> PRT
<213> Mus musculus

<400> 80
Leu Thr Cys Ile Ala Asn Arg Gly Phe Cys Trp His Ser Cys Ile Gln
1 5 10 15

Gly Phe Gln Leu Ala Gly His Cys Gly His Pro Lys Val Arg Leu Leu
20 25 30

His

<210> 81
<211> 34
<212> PRT
<213> Mus musculus

<400> 81
Leu Val Cys Arg Arg Lys Gly Gly Arg Cys Tyr Ile Lys Cys Pro Asp
1 5 10 15

Asn Thr Asp Glx Ile Gly Met Cys Arg Leu Pro Phe Lys Cys Cys Lys
20 25 30

Arg Gln

<210> 82
<211> 34

<212> PRT
<213> Mus musculus

<400> 82
Leu Ser Cys Trp Met Lys Glx Gly Ile Cys Gln Tyr Arg Cys Phe Gly
1 5 10 15

Asn Thr His Lys Ile Gly Ser Cys Gly Ala Pro Phe Leu Lys Cys Cys
20 25 30

Lys Arg